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Epstein–Barr virus status of sporadic Burkitt lymphoma is associated with patient age and mutational features

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Summary

Sporadic Burkitt lymphoma (BL) is the most frequent tumour of children and adolescents but a rare subtype of lymphomas in adults. To date most molecular data have been obtained from lymphomas arising in the young. Recently, Epstein–Barr virus (EBV) positive and negative BL in young patients was shown to differ in molecular features. In the present study, we present a large age-overarching cohort of sporadic BL (n = 162) analysed by immunohistochemistry, translocations of MYC proto-oncogene, basic helix-loop-helix transcription factor (*MYC*), B-cell leukaemia/lymphoma 2 (*BCL2*) and B-cell leukaemia/lymphoma 6 (*BCL6*) and by targeted sequencing. We illustrate an age-associated inter-tumoral molecular heterogeneity in this disease. Mutations affecting inhibitor of DNA binding 3, HLH protein (*ID3*), transcription factor 3 (*TCF3*) and cyclin D3 (*CCND3*), which are highly recurrent in paediatric BL, and expression of sex determining region Y-box transcription factor 11 (SOX11) declined with patient age at diagnosis (P = 0·0204 and P = 0·0197 respectively). In contrast, EBV was more frequently detected in adult patients (P = 0·0262). Irrespective of age, EBV-positive sporadic BL showed significantly less frequent mutations in *ID3*/*TCF3*/*CCND3* (P = 0·0088) but more often mutations of G protein subunit alpha 13 (*GNA13*; P = 0·0368) and forkhead box O1 (*FOXO1*; P = 0·0044) compared to EBV-negative tumours. Our findings suggest that among sporadic BL an EBV-positive subgroup of lymphomas increases with patient age that shows distinct pathogenic features reminiscent of EBV-positive endemic BL.

Keywords: Epstein–Barr virus status, Burkitt lymphoma, age, mutations.

Introduction

Burkitt Lymphoma (BL) is a mature aggressive B-cell lymphoma with a bimodal incidence peak and compared to other mature aggressive B-cell lymphomas, like diffuse large B-cell lymphoma (DLBCL), common in paediatric but infrequent in older-aged adult patients.[1,2] Currently three types of BL are distinguished; which are (i) endemic BL arising predominately in children in central Africa associated with Malaria infection, (ii) immunodeficiency associated BL of patients with various acquired or inherited immune defects and (iii) sporadic BL presenting the most frequent tumour of children worldwide. Most endemic BL and a large proportion of immunodeficiency associated BL carry the

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Epstein–Barr virus (EBV) in the lymphoma cells, whereas the rate of EBV-positivity in sporadic BL varies in geographic regions.[3]

While paediatric BL has been studied molecularly in detail,[4–7] data on adult BLs are sparse. We and others have previously shown molecular features of DLBCL to be associated with patient age, a finding that is of relevance for comparisons of outcome data between age groups or development of age-overarching therapeutic concepts.[8,9] However, age-overarching analysis of BL has been limited to phenotypic studies[10] and small cohorts to date.[11,12] The study by Satou *et al.*[10] suggests that in sporadic BL the association with EBV positivity of the tumour cells increases with patient age. In contrast to BL in adults, endemic BL, a disease predominately of the young, has been studied molecularly in detail and shown to be distinct from sporadic paediatric BL not only by the rate of EBV association but also by molecular features such as mutational load and immunoglobulin V-gene usage.[5,13,14]

Patients and methods

Patient cohort

Initial diagnostic biopsies of paediatric patients (aged ≤18 years) submitted in the years 2001–2013 and adult patients (age >18 years) diagnosed in 2001–2017 with BL were identified in the files of the Hematopathology Section and Lymph Node Registry of the University Hospital Schleswig-Holstein, Campus Kiel. In addition, 13 adult patients with BL were provided from the Departments of Pathology, Robert-Bosch-Krankenhaus, Stuttgart and University of Würzburg. In total we identified 162 patients comprising 92 children and adolescents (≤18 years age at diagnosis, hereafter referred to as paediatric) and 70 adults (>18 years) with BL. All diagnoses were revised according to the 2017 World Health Organization (WHO) Classification.[15] All tissues were formalin fixed and paraffin embedded (FFPE). The study was approved by the Ethics Committee of the Medical Faculty of the University of Kiel (D 429/13) and conducted in accordance with the Declaration of Helsinki. As adult BL were not treated in trials, clinical data were not systematically available. All paediatric BL were registered in trials and/or followed by the Berlin-Frankfurt-Münster-study group (BFM-NHL).

Immunohistochemistry, fluorescence in situ hybridisation (FISH)

The basic immunophenotype was studied using immunohistochemistry with antibodies directed against cluster of differentiation (CD)20, CD10, B-cell leukaemia/lymphoma 2 (BCL2), sex determining region Y-box transcription factor 11 (SOX11) and multiple myeloma oncogene 1 (MUM1). Detection of EBV was performed by EBV-encoded small nuclear RNA (EBER) *in situ* hybridisation. Immunohistochemistry and EBER were performed on an automated stainer using protocols recommended by the supplier as previously published.[16,17] EBV-positivity was defined as the majority of tumour cells being positive. FISH with break-apart and/or double-colour double-fusion probes for the *BCL2*, *BCL6*, and MYC proto-oncogene, basic helix-loop-helix transcription factor (*MYC*) gene loci (Abbott Molecular, Des Plaines, IL, USA; or Zytovision, Bremerhaven, Germany) were used as previously reported.[8] In a subset of cases, FISH results for the t(8;14)(q24;q32) (*MYC-IGH*) translocation were available.

Gene expression profiling

The DNA and RNA were extracted by GeneRead DNA FFPE, AllPrep DNA/RNA FFPE Kit both from Qiagen (Hilden, Germany) or ExpressArt® FFPE Clear RNAready Kit (AmpTec, Hamburg, Germany). Digital multiplexed gene expression (DMGE) classification by nCounter technology was performed as previously described.[18] This assay allows identifying lymphomas with a molecular BL (mBL) signature and lymphomas lacking features of BL (non-mBL). A small group of lymphomas cannot be assigned to mBL or non-mBL by gene expression and are referred to as intermediates.[19]

Mutational analysis

Targeted mutation analysis was performed as described previously using an AmpliSeq Custom Panel that covers 36 genes or mutation 'hotspots' known to be recurrently mutated in aggressive BCL, especially in BL and DLBCL.[20] Only tumoral DNA containing >70% of tumour cells was analysed; corresponding germline tissue was not available thus, could not be analysed for comparison. Genes analysed were: AT-rich interaction domain 1A (*ARID1A*), myocyte enhancer factor 2B (*MEF2B*), caspase recruitment domain family member 11 (*CARD11*), *MYC*, cyclin D3 (*CCND3*), myeloid differentiation factor 88 (*MYD88*), *CD79B*, Notch receptor 1 (*NOTCH1*), cAMP response element-binding protein binding protein (*CREBBP*), *NOTCH2*, C-X-C motif chemokine receptor 4 (*CXCR4*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit D (*PIK3CD*), E1A binding protein P300 (*EP300*), Pim-1 proto-oncogene, serine/threonine kinase (*PIM1*), enhancer of zeste homologue 2 (*EZH2*), PR/SET domain 1 (*PRDM1*), Fas-associated via death domain (*FADD*), phosphatase and tensin homologue (*PTEN*), Fas ligand (*FASLG*), protein tyrosine phosphatase receptor type D (*PTPRD*), forkhead box O1 (*FOXO1*), Ras homologue family member A (*RHOA*), G protein subunit alpha 13 (*GNA13*), splicing factor 3B subunit 1 (*SF3B1*), inhibitor of DNA binding 3, HLH protein (*ID3*), suppressor of cytokine signalling 1 (*SOCS1*), interferon regulatory factor 4 (*IRF4*), Transducin beta-like 1 X-linked receptor 1 (*TBL1XR1*), *IRF8*, transcription factor 3 (*TCF3*), histone-lysine N-methyltransferase 2C (*KMT2C*), tumour necrosis factor alpha-induced protein 3 (*TNFAIP3*), *KMT2D*, tumour

necrosis factor receptor superfamily member 14 (*TNFRSF14*), mitogen-activated protein kinase kinase 1 (*MAP2K1*), tumour protein p53 (*TP53*) (details and analysed regions in Supplementary Table SI).

Samples for sequencing were selected based on the completeness of data and DNA quality/amount. Furthermore, eight samples of normal lymph node tissue were used as panel of normal (PON). Fastq sequencing data analyses were performed using SEQNEXT software (JSI Medical Systems©, Version 5.2.0 build 502). Sequencing data were aligned to the target regions of the panel. A mean of 10 228 252 total reads aligned with a mean read depth of 6004 could be identified. All variants identified in the PON, single-nucleotide polymorphisms (SNPs) with a minor allele frequency ≥1% (dbSNP build 153, 1000 Genomes version r1 and GnomAD version r2.1.1, European, non-finish descent) and variants with a variant allele frequency <15% were excluded from further analyses. To identify potential functional relevant, likely somatic mutations variant effect prediction was done using Ensembl© Variant Effect Predictor (VEP; https://www.ensembl.org/Tools/VEP). Variants were interpreted using SIFT, PolyPhen, FATHMM, Condel, and CLIN_SIG entries and the IMPACT provided by the VEP tool. Variants with benign and/or tolerated effect predictions in all tools and/or low impact were excluded; with the exception of those previously reported to be somatic in BL.[2,21–24] Results were visualised using https://bio.oviz.org/.[25]

Statistics

Immunohistochemistry results for CD20, CD10, BCL2, MUM1, SOX11 and *in situ* hybridisation for EBER were dichotomised as positive or negative using a cut-off of 25% positive lymphoma cells. Statistical analyses, to identify markers significantly associated with age at diagnosis were performed using univariate logistic regression analyses (LRA) as previously described using an R-Scipt[8,9] and all other tests by GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). To analyse the effect of both features, EBV and age on the sample characteristics associated with age, bivariate logistic regression analyses was performed.

Results

Gene expression profiling confirms high diagnostic specificity

Lymphomas identified by the registered diagnosis BL, displaying a morphology compatible with BL, showing expression of CD20 and CD10, Ki67 > 90% and a MYC break or t(8;14) by FISH were included in the study. BCL2 expression was negative or partially/weakly expressed as described to be compatible with BL.[26] Three lymphomas showed slight deviations from the expected phenotype with absence of CD10 in one and absence of CD20 expression in two other lymphomas (Table I). However, as morphology and phenotype except for the aforementioned markers were compatible with BL, these cases were included in the cohort. Lymphomas with chromosomal breaks affecting *BCL2* or *BCL6* were excluded. In only one lymphoma, we did not detect a MYC break due to insufficient hybridisation quality. However, as this case was typical in respect to morphology and immunophenotype, it was included in the final cohort (Table I). In order to understand if our diagnostic selection of BL is contaminated by lymphomas such as DLBCL, digital multiplexed gene expression using nCounter/NanoString was performed as previously described.[18] Of the 61 lymphomas including all adult BL with gene expression being available, none was classified as non-mBL (Table I). The lymphomas classified as intermediate (n = 16) by gene expression profiling formally do not fall into the group of mBL (Table I). However, we have previously shown that typical BL may be classified as intermediate be gene expression due to the predefined cut-offs.[26] Thus, we consider our cohort as a highly pure cohort of BL without contamination by DLBCL or other high-grade B-cell lymphomas.

Mutation analyses identifies highly recurrent mutations typical for BL

Sequencing data were generated for 91 BL specimens, including all adult BL with sufficient DNA (n = 67) and 24 paediatric samples (Fig 1). Overall, 299 different single nucleotide variants and small indels affecting 22 genes or 'hotspot' regions in 88/91 samples (97%) were identified. In three samples (three of 91, 3%) no variant was detected in the targeted approach. Variants were mostly classified as missense mutations (208/299, 70%), stop gains (30/299, 10%) and frameshifts (23/299, 8%). Genes affected by variants in at least 20% of the entire cohort were *ID3* (63%), *MYC* (60%), *TP53* (56%), *CCND3* (45%), and *FOXO1* (23%) (Fig 1). Overall, 57/88 (65%) patients affected by at least one variant, showed variants affecting the *ID3* gene. In 65/88 (74%) of lymphomas variants affecting either *ID3* or *TCF3* and 75/88 (86%) samples carried mutations at least in one of the three genes of the *ID3/TCF3/CCND3* pathway. In all, 11 of the 13 (77%) lymphomas without mutations in *ID3*, *TCF3* or *CCND3* harboured variants affecting other genes known to be mutated in BL such as *GNA13, FOXO1, RHOA, MYC, ARID1A*, and *TP53*.

In our cohort, two lymphomas (two of 88, 2%) carried variants known to occur frequently in mature aggressive B-cell lymphomas of DLBCL type but rarely in BL. One lymphoma (43 years of age at diagnosis, *IG-MYC* positive) displayed a variant effecting *PTEN* together with a variant of *CARD11* but was classified as mBL by gene expression. The second lymphoma (75 years of age at diagnosis) harboured a single variant affecting *EP300*. Unfortunately, gene expression

Table I. Characteristics of the study cohort.

Characteristic	Paediatric, aged 0–18 years	Adult, aged >18 years	Total, aged 0–94 years
Age, years, median (range)	8 (0–17)	55 (20–94)	14 (0–94)
Patients, *n* (%)	92 (57)	70 (43)	162 (100)
Gender, *n/N* (%)			
Male	79/91 (87)	50/70 (71)	129/161 (80)
Female	12/91 (13)	20/70 (29)	32/161 (20)
Immunohistochemistry, *n/N* (%)			
CD10 positive	92/92 (100)	66/67 (99)	158/159 (99)
CD20 positive	91/92 (99)	67/69 (97)	158/160 (99)
MUM1 positive	48/86 (56)	29/60 (48)	77/146 (53)
Fluorescence *in situ* hybridisation, *n/N* (%)			
MYC BA/IGH-*MYC* fusion	91/91 (100)	69/70 (99)	160/161 (99)
BCL2 BA	0/89 (0)	0/66 (0)	0/155 (0)
BCL6 BA	0/85 (0)	0/68 (0)	0/153 (0)
Molecular subtype, *n/N* (%)			
mBL	11/18 (61)	34/43 (79)	45/61 (74)
Non-mBL	0/18 (0)	0/43 (0)	0/61 (0)
Intermediate	7/18 (39)	9/43 (21)	16/61 (26)

BA, break apart; mBL, molecular Burkitt lymphoma; non-mBL, non-molecular Burkitt lymphoma.

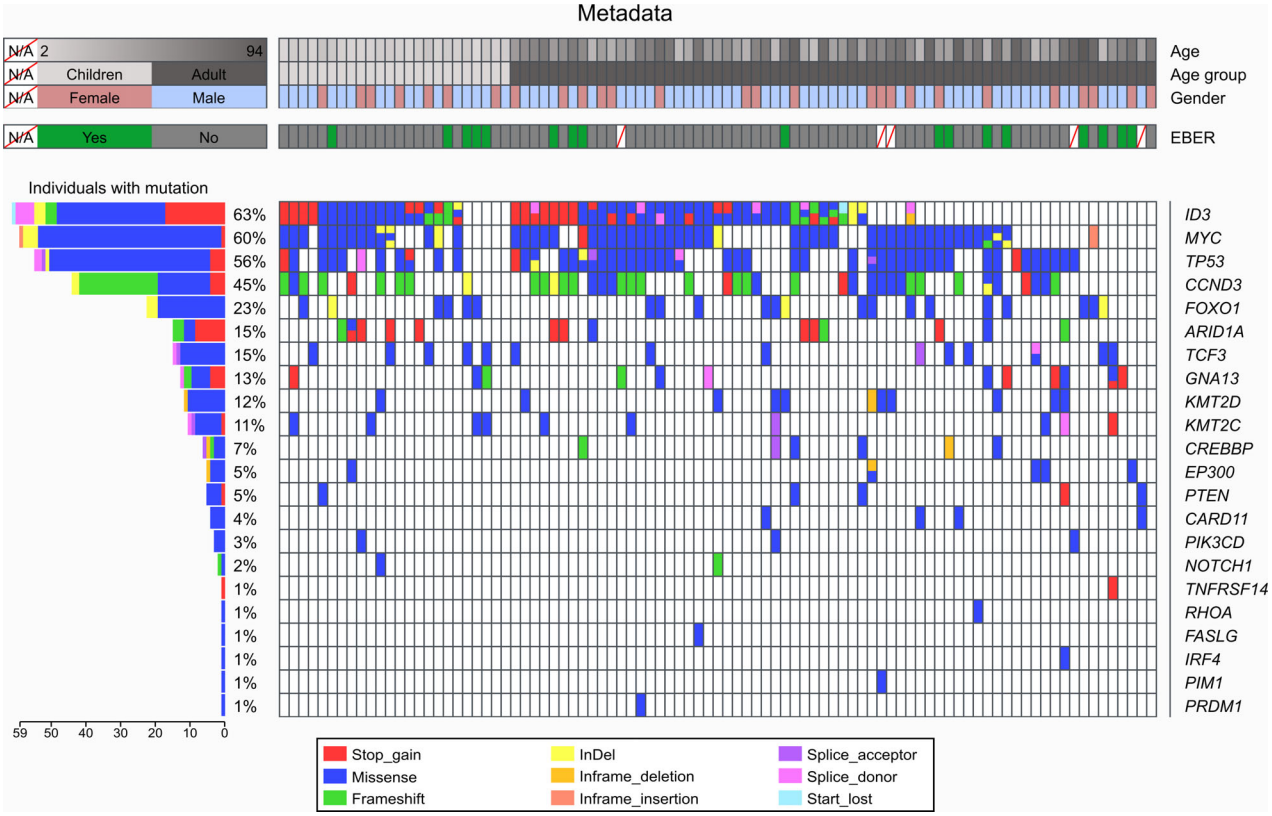

Fig 1. Results of the targeted mutation analyses of paediatric and adult BL sorted according to age group and mutation profile. Visualisation of metadata, EBV status and identified variants of samples analysed by targeted sequencing (https://bio.oviz.org/). Genetic variants are displayed by different colour (red: stop gains, dark blue: missense, green: frameshift: yellow: InDel, orange: inframe deletions, dark orange: inframe insertions, purple: splice acceptor, pink: splice donor and light blue: start lost variants). The bar graph to the left display the mutation frequency and distribution of variants identified. [Colour figure can be viewed at wileyonlinelibrary.com]

was not available for this specimen. However, morphology, *MYC* translocation and phenotype were typical for BL. Overall, we identified variants affecting genes known to be recurrently mutated and distinctive for BL in 98% (86/88) patients. Mutations known to occur in DLBCL are rare or absent in our cohort.

Phenotypic and molecular characteristics of BL associated with age at diagnosis

As expected, gender distribution showed a male predominance in paediatric patients with BL (Table I) and LRA indicates a continuous decrease of male predominance with age at diagnosis ($P = 0{\cdot}0072$, data not shown). The immunophenotype of sporadic BL is known to be rather homogeneous. However, some immunophenotypic markers recognised in BL are variable between specimens, such as BCL2,[26] MUM1,[27] SOX11[28] and the presence of EBV (detected by EBER).[29] SOX11 was expressed significantly more frequently in paediatric BL (32/85, 38%) compared to adults (12/60, 20%) ($P = 0{\cdot}0279$, Fischer's exact test). In addition, the likelihood of SOX11 reactivity in BL significantly decreased with increasing age at diagnosis (LRA, $P = 0{\cdot}0197$, Fig 3). In contrast, EBV was less frequently detected in paediatric compared to adult BL [five of 89 (6%) and 13/65 (20%) respectively, $P = 0{\cdot}0097$, Fischer's exact test]. As expected, the likelihood for a BL to be EBV-positive increased with patient age at diagnosis (LRA, $P = 0{\cdot}0262$, Fig 2). We also detected an increase of BL expressing BCL2 with increasing age, but LRA did not reach statistical significance (Fig 3).

To analyse the age association of somatic mutations, we focussed on genes affected by variants in at least 10% of all BL in our cohort which were *ID3, MYC, TP53, CCND3, FOXO1, ARID1A, TCF3, GNA13, KMT2D,* and *KMT2C*. A gene was considered as affected when at least one variant, passing filtering and VEP was detected in the coding region. Except for *ID3*, no age association of mutations in the aforementioned genes was identified. However, the frequency of variants affecting *ID3* significantly decreased with increasing age at diagnosis (LRA $P = 0{\cdot}0123$, Fig 2C). Similarly, presence of variants targeting either *ID3* and/or *TCF3*, as well as presence of mutations in either *ID3* and/or *TCF3* and/or *CCND3*, were less likely to occur with increasing age at diagnosis (LRA, $P = 0{\cdot}0006$ and $P = 0{\cdot}0204$, respectively, Fig 2).

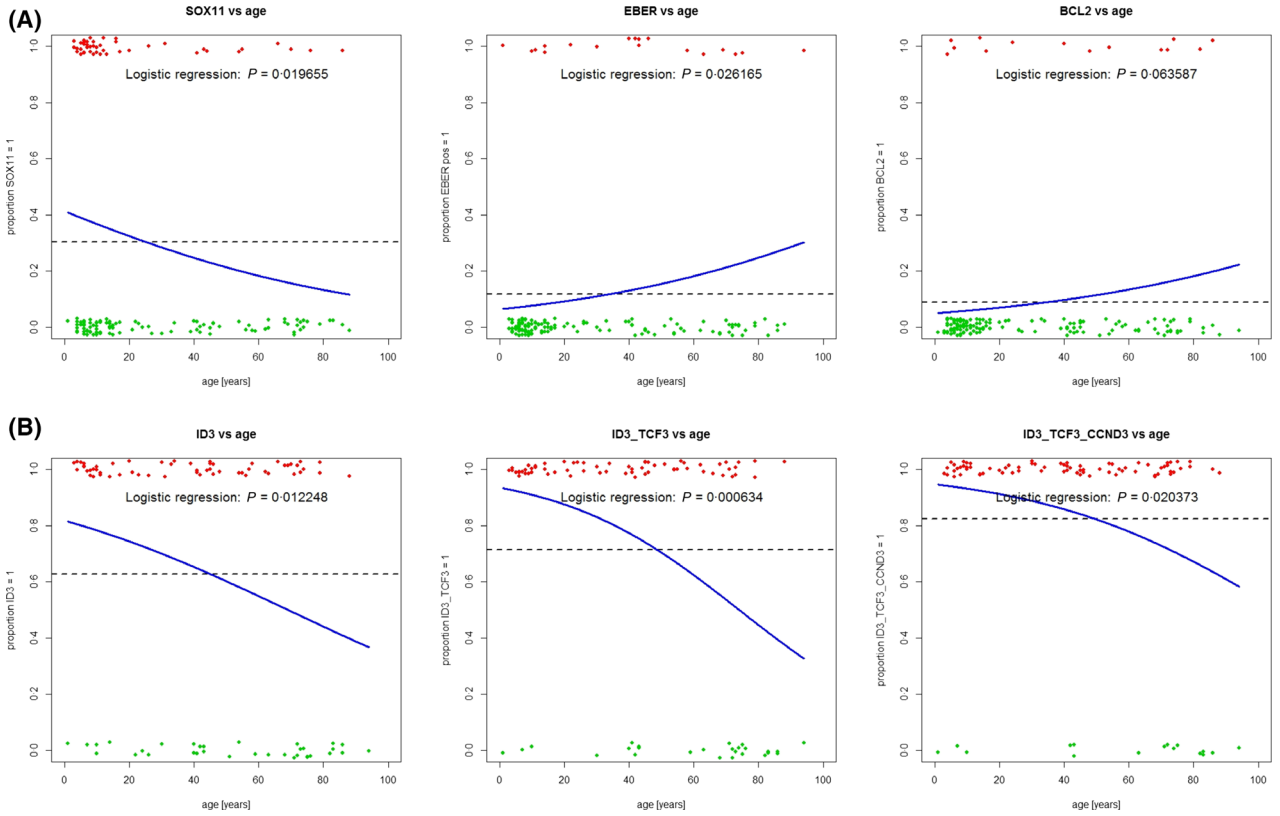

Fig 2. Logistic regression analyses (LRA) of markers showing differences over age in BL, in our cohort. (A) LRA of markers known to be differentially expressed in patients with BL according to age at diagnoses in our study cohort. (B) LRA of the different mutation groups including patients with *ID3* mutations (left panel), with *ID3* or *TCF3* mutations (middle panel) and of patients with either *ID3*, *TCF3* and/or *CCND3* mutations (right panel), that were all significantly associated with younger age at diagnoses. [Colour figure can be viewed at wileyonlinelibrary.com]

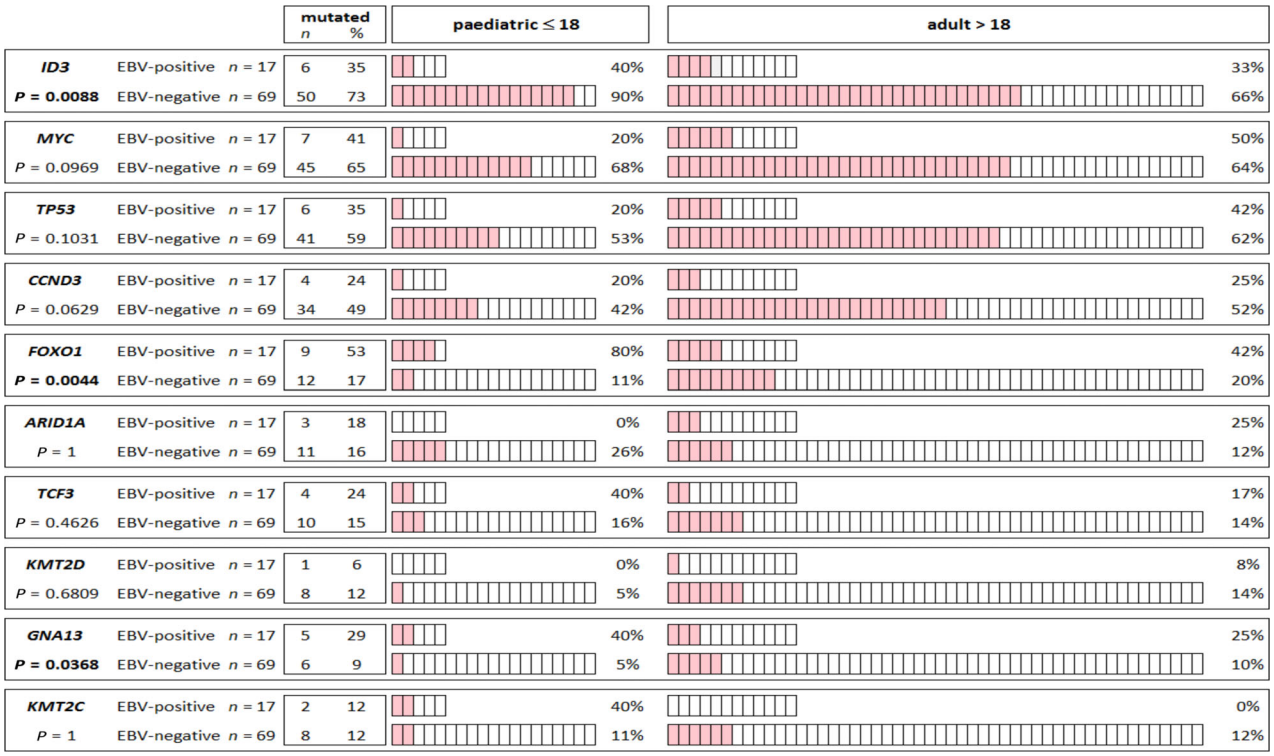

Fig 3. Pattern of variants identified in EBV-positive vs EBV-negative BL at paediatric and adult ages. The pattern of variants identified in the genes altered in at least 10% of BL samples in the overall cohort, separated by age and EBV status are shown. Of 86 BL samples (24 paediatric, 62 adult BL) both EBV status (17 EBV-positive, 69 EBV-negative) and results from the mutation analyses were available. Fisher's exact tests were done on EBV-status and variant frequency in the overall cohort. Significant *P* values (<0·05) are shown in bold. Samples were sorted according to results from the mutation analyses showing samples affected by variants in light red and samples without variants in white. [Colour figure can be viewed at wileyonlinelibrary.com]

EBV-positive sporadic BL are associated with a particular mutation pattern

Given the fact that BL is less likely to be affected by mutations of the *ID3/TCF3/CCND3* pathway with increasing age at diagnosis but more likely to be EBV-positive, we compared the mutational status of EBV-positive and EBV-negative lymphomas. Of note, the cohort was comprised of sporadic BL. EBV-positive sporadic BL significantly more often lacked variants affecting *ID3* but showed an enrichment of *FOXO1* and *GNA13* variants compared to EBV-negative BL ($P = 0{\cdot}0088$, $P = 0{\cdot}0044$, $P = 0{\cdot}0368$, Fisher's exact test, Fig 3). Considering *ID3* and *TCF3* as a complex affecting *CCND3* expression, EBV-positive BL significantly more often lacked mutations in this axis compared to EBV-negative BL [six of 17 (35%) and eight of 69 (12%) respectively, $P = 0{\cdot}0281$, Fisher's exact test]. We also observe a trend of *TP53* mutations to be rarer among EBV-positive than among EBV-negative BL as described previously[5] in paediatric patients but the difference did not reach statistical significance (Fig 3). As shown in Fig 3, the differences in mutation frequency between EBV-positive and –negative BL for *ID3*, occurred in both age groups. Clinical data were available for four of five paediatric patients with EBV-positive BL of which two presented as St. Jude Stage I, one Stage II and one Stage III. None of the patients relapsed.

In order to understand what predicts the mutational pattern better, age or EBV status we included age, EBV status and the mutational status of a given gene together in the LRA. In this bivariate LRA, *ID3* was significantly associated with both, age and EBV status ($P = 0{\cdot}0212$ and $P = 0{\cdot}0045$ respectively). *TCF3* and *CCND3* were not significantly associated with either age or EBV status ($P > 0{\cdot}05$), although the association of *CCND3* with EBV status may indicate a trend ($P = 0{\cdot}0639$). *FOXO1* and *GNA13* were both significantly associated with EBV status ($P = 0{\cdot}0037$ and $P = 0{\cdot}0308$ respectively) but not with age (both $P > 0{\cdot}05$).

No association between SOX11 status and variant status of the genes affected in at least 10% of BL samples could be identified (data not shown). Strikingly, BL classified as intermediate were highly enriched for EBV-positive BLs, compared to BL classified as mBL ($P = 0{\cdot}0024$, Fisher's exact test).

Discussion

Although lymphomas occur in any age group there is a considerable variability of the incidence of distinct entities with age.[30] Moreover, within distinct lymphoma entities molecular,

pathological and clinical features differ between age groups. The latter has been best described for follicular lymphoma[31,32] and DLBCL[9,33] the most frequent B-cell lymphomas in adults. However, an age over-arching analysis for BL, the most frequent B-cell lymphoma in paediatric patients has been sparse to date. To the best of our knowledge we present one of the largest cohorts of sporadic BL analysed for somatic mutations and phenotypic markers. Although we are aware that the lack of matching germline control could lead to false-positive findings whereas the AmpliSeq design could lead to false-negative findings due to allelic-drop-outs or homozygous deletions, we do not think that this affects the conclusions as the same approach was applied to all sub-cohorts.

A unique feature of our present cohort is the age over-arching spectrum of patients allowing us to study age-associated features of BL. Previously published cohorts of BL either contained very few adult patients[4,21,22,34] or were analysed for clinical and phenotypic data only lacking mutational analysis.[10]

As BL and DLBCL may show overlapping pathological features in a subset of cases, we challenged our selection process of cases by a molecular diagnosis based on gene expression profiling that has helped us in previous studies to objectify the diagnosis.[26,35] Moreover, we excluded the most relevant differential diagnosis of Burkitt-like lymphoma with 11q aberrations[36] and high-grade B-cell lymphomas with *MYC* and *BCL2* or *BCL6* translocations (double hit)[37] as far as possible by FISH. Recently, we showed that according to current diagnostic criteria a substantial number of historic diagnoses of BL have to be revised.[38] In line with previous publications, the stringent selection criteria based on commonly available data such as morphology reviewed by experienced pathologists, basic immunophenotype and detection of *MYC* translocations is a very specific approach to separate BL from DLBCL.[20] Although we are confident that our cohort is not contaminated by other lymphomas such as DLBCL, we cannot exclude that our selection process misses a subgroup of mature aggressive B-cell lymphomas pathologically deviating from BL but characterised by an mBL gene expression signature.[1]

The statistical method used in our present study to identify an age association (LRA) adjusts for numbers of patients at a certain age making it unlikely that an enrichment of adult patients in our cohort leads to the effects observed. Anyhow, when we compare our present cohort to all patients diagnosed with BL in the area of Schleswig-Holstein in Northern Germany the proportion of adult patients may even be underrepresented in our study (44%) compared to population-based data (75%, http://www.krebsregister-sh.de/, years 2001–2018, $n = 101$). Thus, we consider it unlikely that our present findings of age association are related to a high number of adult patients in the study cohort.

We describe SOX11 expression to decrease with increasing age at diagnosis, a finding that was not noted in a recent study.[28] However, SOX11 has previously been described as one of the genes to be differentially expressed between BL in children and adults.[12] Whilst the role of SOX11 in the biology of mantle cell lymphoma has been studied in detail and was shown to be involved in differentiation and shaping of the lymphoma microenvironment.[39,40] However, the pathogenic role in BL and precursor cell neoplasms that may both express SOX11 has not yet been studied. Thus, based on current knowledge the relevance of SOX11 in the pathogenesis of BL is uncertain.

In respect to the phenotype we describe that the likelihood of a BL to be positive for EBV increases with age at diagnosis. A similar finding has been previously reported.[10] In line with findings of other EBV-associated lymphomas, the proportion of EBV-positivity of the lymphoma cells is higher in Asian patients[10] compared to our present cohort of patients from central Europe. Nevertheless, we can confirm that in sporadic BL the relative abundance of EBV-associations increased with age at diagnosis. Of note, we only included sporadic BL and no endemic lymphomas, the latter being mostly positive for EBV. However, our data may link previous observations made in EBV-positive mostly endemic BL of paediatric patients[5,13,14] to the effect of age in sporadic BL.[10] EBV-positive sporadic BL were underrepresented in previous studies, which led to the development of a gene expression-based diagnostic tool for BL. Our present data suggest that EBV-positive BL are more often classified as 'intermediate' between mBL and non-mBL despite the fact that all other features (morphology, immunophenotype) are consistent with BL. The data imply that molecular classifiers of BL may need to be adjusted for EBV-positive sporadic BL and future studies are required to investigate how EBV influences expression of classification genes.

In paediatric patients, the presence of EBV-positive BL has been shown to carry substantially less somatic mutations compared to EBV-negative BL, especially in driver mutations and genes involved in apoptosis.[5] Unfortunately, our targeted-sequencing panel does not include all of the genes reported by whole-genome sequencing. Nevertheless, genes of the *ID3/TCF3/CCND3* pathway were among the genes reported to be differentially mutated between EBV-positive and –negative BL in paediatric patients.[5,13,14] The *ID3/TCF3/CCND3* axis is a key pathogenetic pathway in BL,[4,6] but seems to be less frequently mutated in the pathogenesis of BL in the presence of EBV either in the endemic variant[5,13,14] or in elderly patients as shown in our present study. Nevertheless, it might well be that this axis is deregulated by alternative means as recently described by our group, like differential splicing of TCF3 or differential epigenetic regulation.[21,41] It seems not possible to provide a categorical answer to the question what feature drives the mutational status – age or EBV – using our present data set, as both features are closely associated with each other in sporadic BL in central Europe. However, future studies combining age-overriding cohorts of sporadic BL with endemic BL (EBV-positive in young patients) may provide an answer to this question.

Our present data and published data[5,13,14] reinforce the concept to distinguish EBV-positive from EBV-negative BL due to differences in underlying mutational landscape. Although to a much lesser extent compared to DLBCL, our present data suggest that sporadic BL is a disease with age-associated mutational heterogeneity. The treatment of adults with BL is often limited by therapy associated toxicity in elderly patients and thus far less standardised as in children. BL in adults are thus often treated outside of clinical trials. Thus, to what extent the age-associated mutational heterogeneity is clinically relevant needs to be explored in future studies. One might speculate that new therapeutic concepts arising from endemic BL[5] might also be of relevance for BL of elderly when tested positive for EBV. Moreover, features reported to be characteristic for EBV-positive endemic BL such as immunoglobulin V-gene usage[5,14] and EBV-coded protein expression[13] still need to be assessed in EBV-positive BL of elderly patients. And finally, the clinical relevance of this subgroup needs to be determined.

In summary, among sporadic BL the EBV-positive subgroup of lymphomas increases with increasing age of patients at the time of diagnosis. EBV-positive sporadic BL shows distinct mutational features reminiscent of EBV-positive endemic BL.

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Author contributions

Julia Richter, Katharina John, Christian Kohler, Rainer Spang and Wolfram Klapper analysed the data. Annette Staiger, German Ott, Andreas Rosenwald and Sören Franzenburg provided material and/or data. Birgit Burkhardt and Reiner Siebert provided data and logistic support. Ilske Oschlies and Wolfram Klapper reviewed pathology data. Julia Richter and Wolfram Klapper designed the research and wrote the manuscript.

Conflict of interest

Wolfram Klapper has received research funding from Takeda, Regeneron, Amgen not related to the project.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table SI. Overview of regions covered by the AmpliSeq Panel.

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