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Genome Wide Copy Number Analysis of Paediatric Burkitt Lymphoma Using Formalin-Fixed Tissues Reveals a Subset with Gain of Chromosome 13q and Corresponding miRNA Over Expression

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SUMMARY

The majority of paediatric Burkitt lymphoma (pBL) patients that relapse will die of disease, but markers for this high-risk subset are unknown. *MYC* translocations characterize pBL, but additional genetic changes may relate to prognosis and serve as potential biomarkers. We utilized a molecular inversion probe single nucleotide polymorphism assay to perform high resolution, genome-wide copy number analysis on archival formalin-fixed, paraffin-embedded pBL and germline tissues. We identified copy number abnormalities (CNAs) in 18/28 patients (64%) with a total of 62 CNAs that included 32 gains and 30 copy number losses. We identified 7 recurrent CNAs including 1q gain (7/28, 25%), 13q gain (3/28, 11%), and 17p loss (4/28, 14%). The minimum common amplified region on 13q was at 13q31 and included the *MIR17HG* (*MIR17-92*) locus. Samples with this gain had higher levels of *MIR17* RNA and showed a tendency for early relapse. Tumour-specific uniparental disomy was identified in 32% of cases and usually was recurrent. These results demonstrate that high-resolution copy number analysis can be performed on archival lymphoma tissue specimens, which has significance for the study of rare diseases.

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AUTHOR CONTRIBUTIONS

JDS and RRM designed the study, performed the research, analysed the data, and wrote the paper. PDL, VR, MSJ, JMD, and PB performed the research and analysed the data. JNS and PAA performed the research. WGS and SLP performed the research and wrote the paper. MGB provided patient samples and performed the research.

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Keywords

Paediatric Burkitt lymphoma; Copy Number Analysis; Molecular Inversion Probes; Chromosome 13q; MIR17HG; FFPE

INTRODUCTION

Burkitt lymphoma (BL) is a malignancy of mature B-cells that occurs at all ages but is more common in children and accounts for one-third of paediatric non-Hodgkin lymphoma cases (Sandlund, *et al* 1996). Paediatric BL (pBL) is curable in up to 90% of cases, but the majority of the patients that relapse will die of disease (Cairo, *et al* 2007, Patte, *et al* 2007). Thus there is a need to identify prognostic markers that can be used to stratify patients at the time of diagnosis and direct therapy. Although translocation of the *MYC* gene is present in nearly all cases, heterogeneity of tumour genomes in the form of additional gene copy number abnormalities (CNAs) may contribute to the clinical heterogeneity in these patients.

Previous studies of pBL using conventional cytogenetics (Poirel, *et al* 2009) and fluorescence *in situ* hybridization (FISH) (Nelson, *et al* 2009) have identified recurrent genetic abnormalities, including gain of 1q and 13q and loss of 17p, and found that 13q abnormalities are associated with a worse prognosis. Recently, higher resolution genotyping techniques have been applied to BL (not strictly paediatric cases) including comparative genomic hybridization (CGH) (Salaverria, *et al* 2008), array CGH (Toujani, *et al* 2009), and array-based high resolution single nucleotide polymorphism (SNP) analysis (Scholtysik, *et al* 2010). These studies identified recurrent gains of 1q and loss of 17p affecting subsets of BL patients. To date, the combination of high-resolution genotyping and clinical outcomes has not been reported.

Our study focused strictly on pBL with exclusion of BL patients over the age of 18 years. The overall incidence of pBL is quite low, limiting the numbers of cases at each institution, and fresh frozen tissue that is typically required for high-resolution genetic studies is often not available. Therefore, we applied a newer assay that allowed us to utilize formalin-fixed, paraffin-embedded (FFPE) tissues from the pathology archives going back approximately 15 years. We used a genome-wide, high-resolution molecular inversion probe (MIP) SNP assay to identify copy number gains, copy number losses, and uniparental disomy (UPD) on DNA isolated from FFPE pBL diagnostic biopsy tissues. Gain of 13q and loss of 17p were validated by FISH, and microRNA (miRNA) expression levels were assessed to correlate with *MIR17* locus gain on 13q. This represents the first reported high resolution gene copy number analysis performed on lymphoma using FFPE tissues, and we correlate our findings with clinical parameters and outcome available for the majority of the patients. Furthermore, the assessment of germline DNA on most patients enabled us to provide an assessment of UPD in pBL.

METHODS

With approval from the University of Utah Institutional Review Board, 27 pBL cases with adequate available diagnostic biopsy tissue were identified. Five additional cases were obtained from Penn State College of Medicine. The morphology and diagnostic immunohistochemistry and flow cytometry were reviewed by two haematopathologists, and cases with increased nuclear pleomorphism, MIB1<95%, or BCL2 protein expression were excluded to confirm the diagnosis of BL according to the World Health Organization 2008 guidelines (Swerdlow 2008). We applied these stringent criteria because it has been demonstrated that, especially in children, cases with a typical BL morphology and

immunophenotype uniformly show a “molecular BL” expression profile (Dave, *et al* 2006, Hummel, *et al* 2006, Klapper, *et al* 2008). This approach may, however, potentially exclude some cases with a BL gene expression profile but other atypical features.

Full clinical data including age, gender, tumour site, stage, bone marrow involvement, cerebrospinal fluid involvement, therapy group, lactate dehydrogenase (LDH) levels, uric acid levels, and clinical follow-up were obtained from review of medical records for the patients treated at the University of Utah (n=27), while only gender, age, and tumour location were available on the Penn State cases (n=5). 26/27 were treated under the Children’s Oncology Group (COG) 5961 protocol (Cairo, *et al* 2007, Patte, *et al* 2007) and one (BL11) was treated on the high-risk arm of COG 5911 (Cairo, *et al* 2002). One patient (BL22) was lost to follow-up after 32 days and was thus excluded from outcomes analysis.

For 29 of the cases, tumour mass or lymph node biopsies were utilized, and corresponding slides were reviewed to select FFPE blocks with at least 80% tumour, optimal fixation, and minimal necrosis. In 3 cases, the diagnosis was made in a bone marrow specimen, so particle clot sections were utilized to avoid the effects of decalcification: BL16 had 93% tumour cells on aspirate differential and was fixed in B5 rather than formalin; BL32 had 65% tumour cells and was formalin fixed; and BL13 had very scant tissue after cutting and was thus excluded from MIP and polymerase chain reaction (PCR) analysis. Genomic DNA and total RNA were each isolated from a 25 µm thick section using the RecoverAll kit (Applied BioSystems, Foster City, CA).

DNA was quantitated with PicoGreen (Invitrogen, Carlsbad, CA) and submitted for OncoScan FFPE Express (Affymetrix, Santa Clara, CA), a MIP SNP assay, performed at Affymetrix (Schiffman, *et al* 2010, Schiffman, *et al* 2009, Wang, *et al* 2005, Wang, *et al* 2007). The data were then analysed using Nexus Copy Number software (Discovery Edition 5.1, Biodiscovery, El Segundo, CA) with NCBI build 36.1 of the human genome. The SNP-Rank Segmentation Algorithm, a statistically based algorithm similar to the circular binary segmentation (Olshen, *et al* 2004), was used in conjunction with quadratic wave correction. This algorithm applies both the copy number value as well as the B-allele frequency to the segmentation. The significance threshold was set at 5.0^{-7} with a minimum of 5 probes and a maximum contiguous spacing of 1000.0 kbp to define a segment. Copy number gains and losses were set to 0.45 and -0.45 respectively. These settings accommodated the expected heterogeneity of the tumour samples. High gains and big losses indicating greater than a single copy number change were set at 1.2 and -1.2 respectively. Gender correction was applied using Nexus control gender settings. The homozygous frequency threshold was set at 0.95 and the homozygous value threshold was set at 0.8 with a minimum loss of heterozygosity (LOH) requirement of 500 kb. The heterozygous imbalance threshold was set at 0.4. Every gain, loss, or LOH call was directly compared to the patient’s normal sample (when available), and changes present in the germline in areas of copy number variation were rejected. LOH was considered UPD when not associated with copy number loss. Copy number losses mapping to the *IGH@* (14q32.3), *IGK@* (2p11.2), and *IGL@* (22q11.2) immunoglobulin loci were considered due to normal VDJ recombination and not included. Additional calls were rejected in samples with increased noise when there did not appear to be a definitive shift of the baseline.

FISH studies were performed on FFPE tissue sections from nine cases. The LSI® p53 (17p13.1) SpectrumOrange™ DNA Probe (Abbott Molecular, Des Plaines, IL) and BAC probe, which localizes to 13q31.3 (RP11-51B13 and RP11-121J17; Children’s Hospital Oakland Research Institute, Oakland, CA, USA), were utilized to interrogate the loci of interest. For the BAC probes, 1 µg of probe DNA was nick translated with rhodamine-5-dUTP (Enzo Life Sciences, Plymouth Meeting, PA, USA) utilizing a modification of the

manufacturer's protocol (Abbott Molecular). Approximately 200 ng of probe was hybridized and blocked approximately 15 times with a combination of Human Cot-I DNA and human placental DNA (Invitrogen). The SpectrumGreen™ probes specific for CEP® 17 (D17Z1) and LSI® 13q34 were employed as internal controls (Abbott Molecular). Prior to hybridization, slides were pretreated using an automated VP 2000™ processor (Abbott Molecular) with standard protocols. Following pretreatment, the cells and probes were co-denatured at 80°C for 5 min and incubated overnight at 37°C using the HYBrite™ system (Abbott Molecular). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in Antifade solution (Abbott Molecular), and the slides were analysed using Olympus BX61 and Leica DM6000B fluorescence microscopes. Hybridization signals were assessed in 100-400 interphase nuclei per specimen by two individuals. Abnormal ranges for deletion of 17p13.1 and amplification (3-15 copies) of 13q31.3 were $\geq 25\%$ and $\geq 10\%$, respectively. Images were acquired and archived using the CytoVision Image Analysis System (Genetix, San Jose, CA, USA).

Total RNA was quantitated using a NanoDrop 1000 (Thermo Scientific, Waltham, MA), and cDNA was generated using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Quantitative, real-time reverse transcription PCR was performed using the primer-specific TaqMan kit (Applied Biosystems) for *MIR17* reverse transcription (Part #4427975, Assay ID# 2308) followed by qPCR with TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Part #4324018). MiRNA levels were normalized to the expression level of mature miRNA Control *RNU6-2* (*RNU6B*; Part #4427975, Assay ID# 1093). Reactions were performed in triplicate on a Roche LightCycler480, and the results are the mean of two experiments from separate reverse transcription reactions normalized to expression levels from an FFPE spleen specimen. For the assessment of *MYC* mRNA, cDNA was generated with the High Capacity Reverse Transcription kit (Applied Biosystems). Real-time, quantitative PCR was performed on the Roche LightCycler480 using custom-designed primers (available on request) and the SsoFast™ EvaGreen® Supermix (cat#172-5200, Life Science BIO-RAD). Expression was normalized to *HPRT1* and expressed relative to *MYC* transcript levels from FFPE spleen tissue.

Raji and Ramos human BL cell lines were generously provided by Dr. Mitchell Cairo, New York University, and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C with humidified 5% CO₂ atmosphere. RNA was isolated using the RNAqueous® kit (Ambion) and assayed for *MYC* mRNA and *MIR17* as above.

For statistical analysis, pairwise comparisons were made using Fisher's Exact test (for categorical variables) and t-tests (for continuous variables). Survival analysis was performed using the Kaplan-Meier method and log-rank test. Two-tailed p-values are reported, and were considered significant when < 0.05 .

RESULTS

Patient samples and clinical data

Tissue samples were available from 32 pBL patients (Table I). Patients included 25 males and 7 females (M:F ratio 3.6) with a mean age of 7.5 years (range 2 - 18). At presentation, LDH ranged from 443 - 13,851 u/l (median 2,751) and uric acid from 89 - 1909 $\mu\text{mol/l}$ (median 327). Patients were Murphy stage I (n=1), II (n=9), III (n=13), and IV (n=4) and included 1 Group A patient and 4 Group C patients (marrow positive with $> 25\%$ tumour cells); the remainder were Group B. No patients had cerebrospinal fluid disease. Outcomes data were available for 26 patients with a median follow-up of 32.7 months. A total of three patients relapsed (one stage II patient and two stage III patients) and three died (the two stage III patients that relapsed and a different stage II patient who did not have a complete

clinical response). Neither relapse nor survival was significantly associated with stage, therapy group, or LDH greater than twice the upper limit of normal, which may be due to the small sample size.

28 of the 32 tumour samples had adequate tumour tissue and DNA for analysis. BL7 and BL13 were excluded from MIP and PCR analysis when deeper sections revealed a lack of tumour. BL27 and BL31 were excluded from MIP analysis due to poor DNA yields. Negative bone marrows were available for 26 patients, and 24 yielded adequate DNA for MIP analysis (BL11 and BL12 yielded insufficient germline DNA for analysis).

MIP SNP assay

We identified a total of 62 CNAs (defined as change seen in the same cytoband in 1 or more patients) unique to the tumour genome, which included 32 gains and 30 copy number losses (Figure 1; all CNAs and UPD events for each case are listed in the Supplemental Table). 18/28 cases (64%) had at least one gain or loss. Six cases had a single gain or loss, five cases had two, and seven cases had three or more copy number changes. The mean number of gains and losses per case were 1.2 and 1.1, respectively (median 1.0 and 1.0).

In addition, regions of UPD (copy-neutral LOH) were identified in 9 cases (32%) including one case with three UPD segments, three cases with two segments, and five cases with a single UPD segment. UPD segments were often large, involving half or more of the chromosomal arm with a median length of 40.6 MB (range 8.1 – 99.3). Regions with recurrent UPD included 1p (n=4), 1q (n=2), 6p (n=2), 17p (n=3), and 17q (n=2). The only region of UPD that did not occur in more than one sample involved nearly the entire p arm of chromosome 16 in BL28. The median and mean number of UPD segments per case was 1 and 1.6, respectively.

Recurrent CNAs

We identified 7 recurrent CNAs (same cytoband affected in 2 or more patients), which included 2 gains and 5 losses (Table II). We found gains of 1q in 7/28 patients (25%) and gains of 13q in three (11%). Recurrent copy number losses were identified on 17p (n=4, 14%), 13q (n=2, 7%), 14q (n=2, 7%), Xp22.33 (n=3, 11%), and Xp21.3 (n=2, 7%).

The gains on chromosome 1q were generally large, involving almost the entire q arm in three cases (Figure 2A). A minimum common region (MCR) defined by a relatively short 1q gain in BL25 included 7.0 MB localized to cytobands q21.1 - q21.3. However, if this case is ignored, there is a much larger MCR of 48.6 MB from q21.1 to q31.2.

The losses on 17p showed a 4.0 MB MCR from p13.1 to p12 that included 20 genes (Figure 2B). Although this MCR does not include *TP53*, the losses on three of the four cases did include *TP53*. FISH for the 17p13.1 locus was performed on nine cases including three that showed deletion by MIP and six that did not. The three deleted cases were confirmed by FISH while 5/6 negative for copy loss by MIP were negative by FISH. One case was FISH positive (mildly above threshold at 34%) that did not show deletion by MIP. This suggests the possibility of a small tumour subclone in the region where FISH scoring was performed and demonstrates how FISH may detect focal abnormalities that average out copy number across the entire tissue section. Dilution with non-tumour DNA is not likely the cause as other integer copy number changes were distinctly identified at other loci in this sample.

Gain of 13q was identified in 3/28 patients (11%) with a 4.7 MB MCR that includes 3 genes and 8 miRNAs: *GPC5*, *GPC6*, *LINC00410*, *MIR622*, and the *MIR17HG* gene encoding the *MIR17-92* cassette of 7 miRNAs (Figure 2C). Interestingly, two of these cases showed high gains in excess of 5 copies (Figure 1D). Gain of 13q31 was verified by FISH in all three

cases, and no gain was seen in the additional six cases that lacked this gain by MIP. Although the number of patients is small, those with 13q31 gain were more likely to have relapsed over a minimum follow-up period of 6.5 months (2/3 with 13q31 gain relapsed vs. 1/24, $p=0.025$). When formal Kaplan-Meier analysis was performed, the association between 13q31 gain and relapse was not statistically significant ($p=0.19$), most likely due to lack of statistical power. There was no significant relationship between 13q31 gain and stage, therapy group, serum LDH concentration greater than twice the upper limit of normal, or overall survival ($p=0.25$). The patients with gain of 13q31 were also more likely to have loss of 17p with *TP53* deletion (2/3 with 13q31 gain had *TP53* deletion vs. 1/25 without 13q31 gain, $p=0.023$). The association was stronger when patients with *TP53* UPD were included: 3/3 with 3q31 gain had *TP53* deletion or UPD vs. 3/25 without 13q31 gain ($p=0.0061$). However, *TP53* deletion and UPD, either alone or in combination, did not significantly correlate with either relapse ($p=0.71$) or survival ($p=0.75$). The gain of 13q31 was not significantly associated with any other copy number changes; however, the three samples with 13q31 also showed the highest total numbers of copy number and/or UPD events (BL4, $n=10$; BL10, $n=8$; BL25, $n=8$; Supplemental Table). This raises the possibility that 13q31 gain is a marker of overall complexity and that increased genomic changes influence prognosis, but other samples with frequent changes (BL3, $n=7$; BL16, $n=6$; BL19, $n=8$) showed neither 13q31 gain nor clinical events.

MiRNA expression

To examine the effect of 13q31 gain on miRNA expression, we determined the relative expression levels of *MIR17* RNA in all samples (Figure 3A). The samples with 13q31 gain showed higher expression of *MIR17* (mean relative expression of 31.9 in samples with gain vs. 11.7 in those without gain, $p=0.043$; Figure 3B). Two additional samples (BL8 and BL26) showed high *MIR17* levels in the absence of a 13q31 gain suggesting that there are other mechanisms that can lead to increased expression. Because *MIR17HG* is a *MYC* target (O'Donnell, *et al* 2005), we analysed all samples for relative levels of *MYC* transcript expression. Relative *MYC* transcript levels varied from 0.7 to 27.6 (median 8.3) relative to a reactive lymph node control, but *MYC* levels were not elevated the samples with high *MIR17*, and *MIR17* expression showed a slight negative correlation with *MYC* expression (regression slope = -0.4, $R^2=0.05$; Figure 4). For comparison, we also assayed relative *MYC* and *MIR17* in two BL cell lines. The BL cell lines Raji and Ramos showed modestly increased relative *MYC* transcript levels (9.7 and 16.6, respectively) with marked relative increases in *MIR17* miRNA (63.5 and 150.8, respectively). The very high levels of *MIR17* miRNA in the Ramos cells may be related to 13q31 amplification described previously (Karpova, *et al* 2006).

Non-recurrent CNAs

Other notable gains and losses were detected in our cohort but were non-recurrent. One patient, BL1, demonstrated a 400 kb hemizygous deletion of the *SH2D1A* gene at Xq25 in both his tumour and germline DNA, which is diagnostic of X-linked lymphoproliferative disease (XLP). XLP patients are at an approximately 200-fold increased risk for lymphoma, and BL accounts for approximately half of the lymphomas in these patients (Nagy and Klein 2010). This *SH2D1A* deletion may thus represent the cause of this patient's pBL. Deletion of the tumour suppressor gene *FHIT*, which has previously been described in BL (O'Donnell, *et al* 2005, Scholtysik, *et al* 2010), was seen in a single patient (BL4) and was the only gene in the deleted segment. One patient (BL10) demonstrated a high gain of 8q that included the *MYC* locus. Finally, a homozygous 9p21 loss including the *MTAP*, *CDKN2A*, and *CDKN2B* genes was noted in the pBL tumour of a single patient, while UPD involving the *CDKN1A* locus on 6p21 was identified in tumours from two additional patients.

DISCUSSION

We report the first high-resolution (330k) genome-wide copy number analysis of lymphoma performed using FFPE archival tissues. We identified 32 gains and 30 copy number losses with at least one gain or loss in 64% of our samples. Recurrent CNAs were relatively few in number and included two gains and five losses. The incidence of genetic changes is similar to data reported from CGH studies including one study that found aberrant CGH profiles in 17/26 (65%) of classic BL (Salaverria, *et al* 2008) and one that found CNAs in 7/13 patient samples (Toujani, *et al* 2009). A recent high-resolution SNP analysis of 39 frozen BL samples found a total of 308 gains and 220 losses, which probably reflects both higher resolution and a larger number of cases than our current study (Scholtysik, *et al* 2010); this may also reflect the variability of results found when using different copy number calling algorithms in different studies.

The CNAs reported in this study, in particular the recurrent gain of 1q, gain of 13q, and loss of 17p, compare well with what has been described previously by conventional cytogenetics (Lones, *et al* 2004, Poirel, *et al* 2009), FISH (Nelson, *et al* 2009), aCGH (Tagawa, *et al* 2007, Toujani, *et al* 2009), high resolution SNP array (Scholtysik, *et al* 2010), and SNP array analysis of human immunodeficiency virus-related Burkitt lymphoma (Capello, *et al* 2009). Our findings are largely in agreement with the only other high-resolution SNP array study (Scholtysik, *et al* 2010), which showed gains on 1q localized to MCRs at 1q21.1 (26%) and 1q31.3 (33%). They also described 13q31 gain in 18% of samples and 17p13.1 loss in 18%. The recurrent losses we identified at 13q33.1-q34 and Xp22.33 overlap with recurrent MCRs reported in their study, and several non-recurrent gains (4/14) and losses (4/13) that we identified were noted by Scholtysik *et al* (2010). Several recurrent gains reported by Scholtysik *et al.* (2010) that did not occur in our data set include 3q27.3 (*BLC6*, 7/39 samples), 6q15 (7/39), and 7p12.2 (*IKZF1*, 6/39). Of interest, they noted a gain on 18q21.2 in 3/39 cases that included the *POL1* gene and seemed to correlate with increased numbers of CNAs. Although we observed a gain of this region in only one case (BL4), this case did have the largest number of CNAs (6 gains and 4 losses) of any tumour in our study. Overall, the similarities between our findings and previous reports demonstrate the validity of our approach using MIP technology on FFPE specimens.

The *MIR17HG* cluster is oncogenic and has been shown to accelerate lymphoma development in a mouse model (He, *et al* 2005) and increase tumourigenicity in cooperation with *MYC* (Tagawa, *et al* 2007). In addition, expression is increased in lung carcinoma (Hayashita, *et al* 2005) and in colorectal adenoma to adenocarcinoma progression (Diosdado, *et al* 2009) with increased expression linked to both copy number gains in a subset of cases. Increased *MIR17HG* expression has also been shown in mantle cell lymphoma (Bea, *et al* 2009, Navarro, *et al* 2009) and BL (Tagawa, *et al* 2007), and again increased expression was related to copy number changes in some but not all cases. We report a correlation of *MIR17* miRNA expression with CN gain in pBL, which is similar to the results of a previous study that found increased expression of the *MIR17HG* cluster in four BL cases with a 13q31.2 amplification by FISH (Robertus, *et al* 2010). However, additional cases had high level expression of *MIR17* in the absence of 13q31 gain. Although *MIR17HG* has been established as a *MYC* transactivation target in a B-cell line model (O'Donnell, *et al* 2005) and *MIR17HG* expression correlates positively with *MYC* expression in colorectal tumours (Diosdado, *et al* 2009), we were unable to demonstrate a positive association between *MIR17* expression and increased *MYC* mRNA in our patient samples. In addition, the *MIR17* levels were markedly increased in the presence of only modest increases in *MYC* transcripts in two BL cell lines.

We noted a strong correlation between 13q31 gain and loss or UPD on 17p13 that included the *TP53* locus. It is conceivable that the predicted anti-apoptotic effects of *MIR17HG* via targeting *PTEN* and *E2F1* (Hammond 2006) could synergize with the loss of *TP53* function to strongly shift the balance away from apoptosis and towards proliferation in *MYC*-driven tumours. As *TP53* is frequently altered in BL (Gaidano, *et al* 1991), the gain of 13q31 in a smaller subset could lead to more aggressive tumour behaviour. The significance of the association between 13q31 gain and 17p13 loss/UPD merits further investigation and should be confirmed in a larger sample of pBL patients.

Areas of UPD have previously been described in BL (Scholtysik, *et al* 2010) and involve the same chromosome arms as the regions of recurrent UPD in our study: 1p, 1q, 6p, 17p, and 17q. Our study has the advantage of comparison to germline DNA to verify that the UPD events are unique to the tumour genome. The role of these regions of UPD in the pathogenesis of pBL remains to be determined, but the fact that the UPDs tended to be recurrent and that they encompass genes including *CDKN1A* and *TP53* suggest that they may be significant. UPD on 17p correlates with the presence of *TP53* mutations in mantle cell lymphoma (Bea, *et al* 2009), and we have found overexpression of TP53 protein in cases with *TP53* copy loss or UPD, suggesting *TP53* mutation (Leventaki *et al.*, *Mod Pathol* 23:306A, 2011, abstract; manuscript in preparation). UPD has also been described in follicular lymphoma, where UPD of chromosome 1p is associated with a worse prognosis and UPD of 16p is associated with transformation (O'Shea, *et al* 2009). Further study will be required to evaluate the role of UPD in the pathogenesis and prognostication of pBL.

In summary, we report the first high-resolution gene copy number and UPD analysis performed on lymphoma using FFPE tissues. The results of our analysis on pBL FFPE tissues show strong agreement with previous aCGH and SNP array studies performed on frozen BL tissue specimens. In our pBL cohort, gain of 13q31 occurred in 11% and was associated with increased expression of *MIR17*. Although not significantly related to relapse or overall survival by Kaplan-Meier analysis, the categorical association between 13q31 gain and early relapse indicates that further studies in a larger sample set should be performed to assess possible relationships with patient outcomes. Analysis on FFPE greatly expands the ability to perform detailed genetic analysis, which was previously only possible on fresh or frozen tissue samples that are often not available. This approach “unlocks” the FFPE archives and is particularly advantageous for the genomic analysis of rare tumour specimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

- Bea S, Salaverria I, Armengol L, Pinyol M, Fernandez V, Hartmann EM, Jares P, Amador V, Hernandez L, Navarro A, Ott G, Rosenwald A, Estivill X, Campo E. Uniparental disomies, homozygous deletions, amplifications, and target genes in mantle cell lymphoma revealed by integrative high-resolution whole-genome profiling. *Blood*. 2009; 113:3059–3069. [PubMed: 18984860]

- Cairo MS, Krailo MD, Morse M, Hutchinson RJ, Harris RE, Kjeldsberg CR, Kadin ME, Radel E, Steinherz LJ, Morris E, Finlay JL, Meadows AT. Long-term follow-up of short intensive multiagent chemotherapy without high-dose methotrexate ('Orange') in children with advanced non-lymphoblastic non-Hodgkin's lymphoma: a children's cancer group report. *Leukemia*. 2002; 16:594–600. [PubMed: 11960338]
- Cairo MS, Gerrard M, Sposto R, Auperin A, Pinkerton CR, Michon J, Weston C, Perkins SL, Raphael M, McCarthy K, Patte C. Results of a randomized international study of high-risk central nervous system B non-Hodgkin lymphoma and B acute lymphoblastic leukemia in children and adolescents. *Blood*. 2007; 109:2736–2743. [PubMed: 17138821]
- Capello D, Scandurra M, Poretti G, Rancoita PM, Mian M, Gloghini A, Deambrogi C, Martini M, Rossi D, Greiner TC, Chan WC, Ponzoni M, Moreno SM, Piris MA, Canzonieri V, Spina M, Tirelli U, Inghirami G, Rinaldi A, Zucca E, Favera RD, Cavalli F, Larocca LM, Kwee I, Carbone A, Gaidano G, Bertoni F. Genome wide DNA-profiling of HIV-related B-cell lymphomas. *British Journal of Haematology*. 2009; 148:245–255. [PubMed: 19832807]
- Dave SS, Fu K, Wright GW, Lam LT, Kluin P, Boerma EJ, Greiner TC, Weisenburger DD, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Delabie J, Rimsza LM, Braziel RM, Grogan TM, Campo E, Jaffe ES, Dave BJ, Sanger W, Bast M, Vose JM, Armitage JO, Connors JM, Smeland EB, Kvaloy S, Holte H, Fisher RI, Miller TP, Montserrat E, Wilson WH, Bahl M, Zhao H, Yang L, Powell J, Simon R, Chan WC, Staudt LM. Molecular diagnosis of Burkitt's lymphoma. *New England Journal of Medicine*. 2006; 354:2431–2442. [PubMed: 16760443]
- Diosdado B, van de Wiel MA, Terhaar Sive Droste JS, Mongera S, Postma C, Meijerink WJ, Carvalho B, Meijer GA. MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. *British Journal of Cancer*. 2009; 101:707–714. [PubMed: 19672269]
- Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM, Dalla-Favera R. p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences U S A*. 1991; 88:5413–5417.
- Hammond SM. MicroRNAs as oncogenes. *Current Opinion in Genetics and Development*. 2006; 16:4–9. [PubMed: 16361094]
- Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Research*. 2005; 65:9628–9632. [PubMed: 16266980]
- He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. *Nature*. 2005; 435:828–833. [PubMed: 15944707]
- Hummel M, Bentink S, Berger H, Klapper W, Wessendorf S, Barth TF, Cogliatti SB, Dierlamm J, Feller AC, Hansmann ML, Haralambieva E, Harder L, Hasenclever D, Kuhn M, Lenze D, Lichter P, Martin-Subero JI, Moller P, Muller-Hermelink HK, Ott G, Parwaresch RM, Pott C, Rosenwald A, Rosolowski M, Schwaenen C, Sturzenhovecker B, Szczepanowski M, Trautmann H, Wacker HH, Spang R, Loeffler M, Trumper L, Stein H, Siebert R. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *New England Journal of Medicine*. 2006; 354:2419–2430. [PubMed: 16760442]
- Karpova MB, Schoumans J, Blennow E, Ernberg I, Henter JI, Smirnov AF, Nordenskjold M, Fadeel B. Combined spectral karyotyping, comparative genomic hybridization, and in vitro apoptotyping of a panel of Burkitt's lymphoma-derived B cell lines reveals an unexpected complexity of chromosomal aberrations and a recurrence of specific abnormalities in chemoresistant cell lines. *International Journal of Oncology*. 2006; 28:605–617. [PubMed: 16465364]
- Klapper W, Szczepanowski M, Burkhardt B, Berger H, Rosolowski M, Bentink S, Schwaenen C, Wessendorf S, Spang R, Moller P, Hansmann ML, Bernd HW, Ott G, Hummel M, Stein H, Loeffler M, Trumper L, Zimmermann M, Reiter A, Siebert R. Molecular profiling of pediatric mature B-cell lymphoma treated in population-based prospective clinical trials. *Blood*. 2008; 112:1374–1381. [PubMed: 18509088]

- Leventaki V, Rodic V, Jahromi MS, Downie JM, Tripp SR, Bayerl MG, Perkins SL, Barnette P, Schiffman JD, Miles RR. The p53 Pathway is Frequently Deregulated in Pediatric Burkitt Lymphoma, but Over Expression of MDM2 or HDM4 is Not a Common Mechanism. *Modern Pathology*. 2011; 24:306A.
- Lones MA, Sanger WG, Le Beau MM, Heerema NA, Sposto R, Perkins SL, Buckley J, Kadin ME, Kjeldsberg CR, Meadows A, Siegel S, Finlay J, Bergeron S, Cairo MS. Chromosome abnormalities may correlate with prognosis in Burkitt/Burkitt-like lymphomas of children and adolescents: a report from Children's Cancer Group Study CCG-E08. *Journal of Pediatric Hematology/Oncology*. 2004; 26:169–178. [PubMed: 15125609]
- Nagy N, Klein E. Deficiency of the proapoptotic SAP function in X-linked lymphoproliferative disease aggravates Epstein-Barr virus (EBV) induced mononucleosis and promotes lymphoma development. *Immunology Letters*. 2010; 130:13–18. [PubMed: 20080127]
- Navarro A, Bea S, Fernandez V, Prieto M, Salaverria I, Jares P, Hartmann E, Mozos A, Lopez-Guillermo A, Villamor N, Colomer D, Puig X, Ott G, Sole F, Serrano S, Rosenwald A, Campo E, Hernandez L. MicroRNA expression, chromosomal alterations, and immunoglobulin variable heavy chain hypermutations in Mantle cell lymphomas. *Cancer Research*. 2009; 69:7071–7078. [PubMed: 19690137]
- Nelson M, Perkins SL, Dave BJ, Coccia PF, Bridge JA, Lyden ER, Heerema NA, Lones MA, Harrison L, Cairo MS, Sanger WG. An increased frequency of 13q deletions detected by fluorescence in situ hybridization and its impact on survival in children and adolescents with Burkitt lymphoma: results from the Children's Oncology Group study CCG-5961. *British Journal of Haematology*. 2009; 148:600–610. [PubMed: 19895612]
- O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*. 2005; 435:839–843. [PubMed: 15944709]
- O'Shea D, O'Riain C, Gupta M, Waters R, Yang Y, Wrench D, Gribben J, Rosenwald A, Ott G, Rimsza LM, Holte H, Cazier JB, Johnson NA, Campo E, Chan WC, Gascoyne RD, Young BD, Staudt LM, Lister TA, Fitzgibbon J. Regions of acquired uniparental disomy at diagnosis of follicular lymphoma are associated with both overall survival and risk of transformation. *Blood*. 2009; 113:2298–2301. [PubMed: 19141865]
- Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. *Biostatistics*. 2004; 5:557–572. [PubMed: 15475419]
- Patte C, Auperin A, Gerrard M, Michon J, Pinkerton R, Sposto R, Weston C, Raphael M, Perkins SL, McCarthy K, Cairo MS. Results of the randomized international FAB/LMB96 trial for intermediate risk B-cell non-Hodgkin lymphoma in children and adolescents: it is possible to reduce treatment for the early responding patients. *Blood*. 2007; 109:2773–2780. [PubMed: 17132719]
- Poirel HA, Cairo MS, Heerema NA, Swansbury J, Auperin A, Launay E, Sanger WG, Talley P, Perkins SL, Raphael M, McCarthy K, Sposto R, Gerrard M, Bernheim A, Patte C. Specific cytogenetic abnormalities are associated with a significantly inferior outcome in children and adolescents with mature B-cell non-Hodgkin's lymphoma: results of the FAB/LMB 96 international study. *Leukemia*. 2009; 23:323–331. [PubMed: 19020548]
- Robertus JL, Kluiver J, Weggemans C, Harms G, Reijmers RM, Swart Y, Kok K, Rosati S, Schuurin E, van Imhoff G, Pals ST, Kluin P, van den Berg A. MiRNA profiling in B non-Hodgkin lymphoma: a MYC-related miRNA profile characterizes Burkitt lymphoma. *British Journal of Haematology*. 2010; 149:896–899. [PubMed: 20331457]
- Salaverria I, Zettl A, Bea S, Hartmann EM, Dave SS, Wright GW, Boerma EJ, Kluin PM, Ott G, Chan WC, Weisenburger DD, Lopez-Guillermo A, Gascoyne RD, Delabie J, Rimsza LM, Braziel RM, Jaffe ES, Staudt LM, Muller-Hermelink HK, Campo E, Rosenwald A. Chromosomal alterations detected by comparative genomic hybridization in subgroups of gene expression-defined Burkitt's lymphoma. *Haematologica*. 2008; 93:1327–1334. [PubMed: 18698080]
- Sandlund JT, Downing JR, Crist WM. Non-Hodgkin's lymphoma in childhood. *New England Journal of Medicine*. 1996; 334:1238–1248. [PubMed: 8606720]
- Schiffman JD, Wang Y, McPherson LA, Welch K, Zhang N, Davis R, Lacayo NJ, Dahl GV, Faham M, Ford JM, Ji HP. Molecular inversion probes reveal patterns of 9p21 deletion and copy number

- aberrations in childhood leukemia. *Cancer Genetics and Cytogenetics*. 2009; 193:9–18. [PubMed: 19602459]
- Schiffman JD, Hodgson JG, VandenBerg SR, Flaherty P, Polley MY, Yu M, Fisher PG, Rowitch DH, Ford JM, Berger MS, Ji H, Gutmann DH, James CD. Oncogenic BRAF mutation with CDKN2A inactivation is characteristic of a subset of pediatric malignant astrocytomas. *Cancer Research*. 2010; 70:512–519. [PubMed: 20068183]
- Scholtysik R, Kreuz M, Klapper W, Burkhardt B, Feller AC, Hummel M, Loeffler M, Rosolowski M, Schwaenen C, Spang R, Stein H, Thorns C, Trumper L, Vater I, Wessendorf S, Zenz T, Siebert R, Kupperts R. Detection of genomic aberrations in molecularly defined Burkitt's lymphoma by array-based, high resolution, single nucleotide polymorphism analysis. *Haematologica*. 2010; 95:2047–2055. [PubMed: 20823134]
- Swerdlow, SH.; Campo, E.; Harris, NL.; Jaffe, ES.; Pileri, SA.; Stein, H.; Thiele, J.; Vardiman, JW., editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. IARC Press; Lyon, France: 2008.
- Tagawa H, Karube K, Tsuzuki S, Ohshima K, Seto M. Synergistic action of the microRNA-17 polycistron and Myc in aggressive cancer development. *Cancer Science*. 2007; 98:1482–1490. [PubMed: 17608773]
- Toujani S, Dessen P, Ithzar N, Danglot G, Richon C, Vassetzky Y, Robert T, Lazar V, Bosq J, Da Costa L, Perot C, Ribrag V, Patte C, Wiels J, Bernheim A. High resolution genome-wide analysis of chromosomal alterations in Burkitt's lymphoma. *PLoS One*. 2009; 4:e7089. [PubMed: 19759907]
- Wang Y, Moorhead M, Karlin-Neumann G, Falkowski M, Chen C, Siddiqui F, Davis RW, Willis TD, Faham M. Allele quantification using molecular inversion probes (MIP). *Nucleic Acids Research*. 2005; 33:e183. [PubMed: 16314297]
- Wang Y, Moorhead M, Karlin-Neumann G, Wang NJ, Ireland J, Lin S, Chen C, Heiser LM, Chin K, Esserman L, Gray JW, Spellman PT, Faham M. Analysis of molecular inversion probe performance for allele copy number determination. *Genome Biology*. 2007; 8:R246. [PubMed: 18028543]

Figure 1.

Summary of copy number gains and losses. Gains are indicated by the bars to the right of each chromosome, and losses are shown to the left. The thicker bars indicate areas of recurrent change.

Figure 2.

Alignments are shown for recurrent gain of chromosome 1q (A), recurrent loss of chromosome 17p (B), and recurrent gain of chromosome 13q (C). D shows the probe distribution and gain amplitude for the three samples with 13q31 gain.

Figure 3.

Relative expression of *MIR17* miRNA is higher in samples with 13q31 gain. (A) Expression was measured by RT-PCR and is expressed as a ratio of mature miRNA control *RNU6-2* expression for each sample. The samples with 13q31 *MIR17HG* locus gain are indicated by the cross-hatched fill. Results are the mean of two experiments from separate reverse transcription reactions normalized to expression levels from an FFPE spleen specimen. (B) The mean expression of *MIR17* miRNA is significantly higher in samples with gain of 13q31; * $p=0.043$, t-test.

Figure 4.

Comparison of relative expression of *MYC* and *MIR17*. The relative expression level of *MIR17* is plotted for each sample as a function of the relative level of *MYC*, and there is no significant correlation.

Patient demographics and clinical characteristics. The event column shows relapse and/or death with the total months of follow up.

Table 1

Sample ID	M/F	Age (years)	LDH (u/l)	Murphy Stage	Therapy Group	Primary Location	Event (follow up, months)
BL1	M	6.0	4712	III	B	retroperitoneum	None (21.9)
BL2	M	5.1	2064	II	B	pelvis	Death (2.5)
BL3	M	9.6	4868	III	B	abdomen	None (23.6)
BL4	M	10.8	606	II	B	neck	None (21.4)
BL5	M	6.7	1202	II	B	abdomen	None (37.9)
BL6	F	2.4	875	III	B	maxillary sinus	None (27.5)
BL7*	M	8.3	1207	III	B	neck	None (40.0)
BL8	F	7.0	983	III	B	neck	None (54.5)
BL9	M	5.7	510	II	B	caecum	None (59.6)
BL10	M	11.3	1535	III	B	appendix	Relapse/Death (4.2)
BL11	M	4.0	1026	III	B	bowel, peritoneal fluid	None (139.3)
BL12	M	6.9	850	III	B	pharynx	None (141.4)
BL13*	M	8.1	7155	IV	C	bone marrow	None (120.8)
BL15	M	6.6	3243	III	B	abdomen	None (66.3)
BL16	M	8.0	13851	IV	C	abdomen	None (90.6)
BL17	F	11.7	4491	IV	C	abdomen	None (69.2)
BL18	F	11.8	3583	III	B	abdomen	Relapse (4.2) Death (7.3)
BL19	M	11.1	1809	III	B	abdomen	None (64.0)
BL20	M	5.3	576	II	B	ileocaecal, appendix	None (65.8)
BL21	F	2.1	10100	II	B	bowel	None (23.1)
BL22 [#]	M	3.5	504	III	B	ileocaecal	Lost to F/U (1.1)
BL23	F	13.8	443	II	B	neck	None (106.1)
BL24	F	2.0	949	I	B	maxilla	None (12.2)
BL25	M	15.2	858	II	A	ileocaecal	Relapse (2.5) Death (13.4)
BL26	M	11.9	1394	II	B	pelvis	None (12.1)

Sample ID	M/ F	Age (years)	LDH (u/l)	Murphy Stage	Therapy Group	Primary Location	Event (follow up, months)
BL27*	M	16.0	N/A	N/A	N/A	omentum	N/A
BL28	M	18.0	N/A	N/A	N/A	bone	N/A
BL29	M	6.0	N/A	N/A	N/A	ileum	N/A
BL30	M	6.0	N/A	N/A	N/A	abdomen	N/A
BL31*	M	10.0	N/A	N/A	N/A	neck	N/A
BL32	M	7.0	5380	IV	C	retroperitoneum, bone marrow	None (11.8)
BL33	M	9.0	2178	III	B	small bowel	None (6.5)

* Samples excluded from MIP analysis due to insufficient tissue (BL13), insufficient DNA (BL27 and BL31) or lack of tumour in deeper sections (BL7). BL14 was excluded up front as the morphology was most consistent with diffuse large B-cell lymphoma.

Patient BL22 was excluded from survival analysis due to a very short time before the patient was lost to follow-up.

Table II

Recurrent CNAs and UPD events. This table shows the events that overlapped in at least two samples. The start and stop coordinates of the event are according to the NCBI build 36.1 of the human genome.

RECURRENT GAINS									
Chromosome	Samples	MCR	Start	Stop	Selected Genes				
1	BL3, 16, 18, 22, 25, 26, 29	q21.1 - q21.3	143,323,521	150,356,270	<i>BCL9, MLLT11, NOTCH2NL</i> , numerous histone genes				
1	BL3, 16, 18, 22, 26, 29	q21.1 - q31.2	143,323,521	191,925,150	Larger MCR ignoring BL25 includes most of 1q				
13	BL4, 10, 25	q31.3 - q32.1	89,141,212	93,878,941	<i>GPC5, GPC6, LINC00410, MIR17HG</i>				
RECURRENT LOSSES									
Chromosome	Samples	MCR	Start	Stop	Selected Genes				
17	BL4, 10, 19, 25	p13.1 - p12	9,835,232	13,847,301	20 genes, 2 miRNAs (<i>TP53</i> included in BL4,19,25)				
13	BL4, 10	q33.1 - q34	101,132,918	114,142,980	60 genes; <i>FGF14, GAS6, SOX1</i>				
14	BL3, 16	q11.2	21,696,556	21,988,932	No genes				
X	BL19, 24, 29	p22.33	2,713,391	3,826,133	9 genes; 4 Arylsulfatases				
X	BL19, 24	p21.3	26,511,065	27,744,90	<i>DCAF8L2, MAGEB10, SMEK3P</i>				
RECURRENT UPD									
Chromosome	Samples	MCR	Start	Stop	Selected Genes				
1	BL3, 5, 16, 25	p36.33 - p34.2	0	41,284,030					
1	BL12, 22	q42.13 - q44	226,774,825	247,249,719					
6	BL5, 28	p25.3 - p21.2	0	38,324,157	<i>CDKN1A, IRF4</i>				
17	BL5, 10, 12	p13.3 - 13.1	0	8,055,205	<i>TP53</i> ; Overlaps with loss on BL4, 19, and 25				
17	BL8, 25	q21.31 - q25.3	38,795,009	78,774,742					