Published in final edited form as:

Br J Haematol. 2010 February ; 148(4): 600–610. doi:10.1111/j.1365-2141.2009.07967.x.

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

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Supplemental material

A complete list of the members of the Children's Cancer Group for this article is available online:

Appendix: COG (institution by alphabetic order). A. B. Chandler Medical Center, University of Kentucky; Albany Medical Center; Allan Blair Cancer Centre; Atlantic Health System; Baystate Medical Center; British Columbia's Children's Hospital; Brooklyn Hospital Center; C. S. Mott Children's Hospital; Cabell Huntington Hospital; CancerCare Manitoba; Cedars-Sinai Medical Center; Children's Healthcare of Atlanta, Emory University; Children's Hem/Onc Team at Covenant Children's Hospital; Children's Hospital at the Medical Center of Central Georgia; Children's Hospital and Clinics Minneapolis and St Paul; Children's Hospital and Regional Medical Center; Children's Hospital Central California; Children's Hospital Los Angeles; Children's Hospital Medical Center Cincinnati; Children's Hospital Medical Center-Akron, Ohio; Children's Hospital Oakland; Children's Hospital of Austin; Children's Hospital of Orange County; Children's Hospital of Philadelphia; Children's Hospital of Pittsburgh; Children's Hospital-King's Daughters; Children's Medical Center Dayton; Children's Memorial Hospital of Omaha; Children's National Medical Center-D.C.; Christiana Care Health Services/A. I. duPont Institute; City of Hope National Medical Center; Clarian Health; Columbia Presbyterian College of Physicians and Surgeons; Columbus Children's Hospital; Connecticut Children's Medical Center; Cooper Hospital/University Medical Center; Dakota Clinic; Deaconess Medical Center; DeVos Children's Hospital; Doernbecher Children's Hospital; East Tennessee Children's Hospital; Geisinger Medical Center; Georgetown University Medical Center; Group Health Cooperative; Gundersen Lutheran; Henry Ford Hospital; Indiana University-Riley Children's Hospital; IWK Health Centre; Janeway Child Health Center; Kaiser Permanente Medical Group, Northern CA; Kalamazoo Center for Medical Studies; Kosair Children's Hospital; Loma Linda University Medical Center; Loyola University Medical Center; Lutheran General Children's Medical Center; M. D. Anderson Cancer Center; Marshfield Clinic; Mary Bridge Hospital; Mayo Clinic and Foundation; Medical College of Georgia Children's Medical Center; Mercy Children's Hospital; MeritCare Medical Group DBA Roger Maris Cancer Center; Michigan State University; Miller Children's Hospital/Harbor; Montefiore Medical Center; Mountain States Tumor Institute; New York Hospital-Cornell University Medical Center; New York University Medical Center; Newark Beth Israel Medical Center; Penn State Children's Hospital, Hershey Med Center; Phoenix Children's Hospital; Presbyterian/St Luke's Medical Center and CHOA; Primary Children's Medical Center; Princess Margaret Hospital for Children; Quain and Ramstad Clinic; Rainbow Babies and Children's Hospital; Raymond Blank Children's Hospital; Saint Barnabas Medical Center; Santa Barbara Cottage Children's Hospital; Saskatoon Cancer Center; Schneider Children's Hospital; Sinai Hospital of Baltimore; Sioux Valley Children's Specialty Clinics; South Carolina Cancer Center; Southern California Permanente Medical Group; Southern Illinois University School of Medicine; St. Joseph's Hospital and Medical Center; Sunrise Children's Hospital, Sunrise Hospital and Medical Center; Sydney Children's Hospital; Texas Tech UHSC, Amarillo; The Children's Hospital, Denver, CO; The Children's Hospital at The Cleveland Clinic; The Children's Mercy Hospital; The University of Chicago Comer Children's Hospital; UCLA School of Medicine; UCSF School of Medicine; University of Illinois, Rockford; University of Iowa Hospitals and Clinics; University of Medicine and Dentistry of New Jersey; University of Minnesota Cancer Center; University of Nebraska Medical Center; University of North Carolina at Chapel Hill; University of Wisconsin, Children's Hospital, Madison; Vanderbilt Children's Hospital; William Beaumont Hospital; Winthrop University Hospital.

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Summary

Burkitt lymphoma (BL), an aggressive B-cell malignancy, is often curable with short intensive treatment regimens. Nearly all BLs contain rearrangements of the *MYC*/8q24 region; however, recent cytogenetic studies suggest that certain secondary chromosomal aberrations in BL correlate with an adverse prognosis. In this multi-center study, the frequency and impact on clinical outcome of del(13q) and +7 in addition to *MYC* rearrangements as detected by fluorescence in situ hybridization (FISH) in children and adolescents with intermediate and high-risk BL registered on Children's Cancer Group study CCG-5961 were investigated. Analysis with 13q14.3 and 13q34 loci specific probes demonstrated deletions of 13q in 38/90 (42%) cases. The loss of either 13q14.3 or 13q34 alone occurred in 14% and 8%, respectively, while 20% exhibited loss of both regions. Gain of chromosome 7 was observed in 7/68 (10%) cases and *MYC* rearrangements were detected in 84/90 (93%). Prognostic analysis controlling for known risk factors demonstrated that patients exhibiting loss of 13q, particularly 13q14.3, had a significant decrease in 5-year overall survival (77% vs. 95%, $p=0.012$). These observations indicate that del(13q) occurs in childhood BL at frequencies higher than previously detected by classical cytogenetics and underscores the importance of molecular cytogenetics in risk stratification.

Keywords

Burkitt lymphoma; chromosome 13 deletion; fluorescence in situ hybridization; FISH; survival

Introduction

Burkitt lymphoma/leukemia (BL), though rare in adults, accounts for 30-50% of non-Hodgkin lymphoma (NHL) in children and adolescents less than 21 years of age in non-endemic areas (Sandlund *et al*, 1996; Leocini *et al*, 2008). BL is an aggressive mature B-cell malignancy but the prognosis for many patients has greatly improved with the introduction of short, intensive chemotherapeutic regimens (Link *et al*, 1997; Reiter *et al*, 1999; Gerrard *et al*, 2008; Cairo *et al*, 2007; Patte *et al*, 2007). Due to its rapid growth pattern (high growth fraction and doubling time between 24 and 48 hour (Leocini *et al*, 2008)) the initial treatments developed for BL were intensive. Recent clinical studies have shown that low risk patients with resected localized disease have an excellent prognosis with cure rates nearing 100% (4 year event-free survival [EFS] of 98.3% and overall survival [OS] of 99.2%) and minimal toxicity following short, low intensity treatments (Gerrard *et al*, 2008). Children and adolescents with intermediate and high-risk BL also have a good prognosis with OS ranging from 82-93% (Cairo *et al*, 2007; Patte *et al*, 2007). A recent FAB/LMB96 (French American British/Lymphomas Malins B) trial demonstrated that reductions in treatment were successful not only in patients with less advanced stages, but also in early responding patients with central nervous system (CNS)-negative advanced stage and with high lactate dehydrogenase (LDH) levels, thereby potentially decreasing the risk for medication-induced toxicities (Patte *et al*, 2007). Combined bone marrow and CNS disease and inadequate response to reduction therapy continue to be associated with decreased EFS (Cairo *et al*, 2007; Patte *et al*, 2007). In order to allow for reduction in

therapy and therapy-related toxicity, much interest has focused on identification of additional prognostic factors at the time of diagnosis for patients with BL.

BL is a unique hematological malignancy, but an accurate diagnosis is dependent upon close correlation of the histologic, immunophenotypic and genetic features. One of the hallmark genetic features of all clinical and morphologic BL subtypes is chromosomal rearrangement of the *MYC* gene region at 8q24 (Leoncini *et al*, 2008; Kaiser-McCaw *et al*, 1977). The most common translocation, occurring in approximately 70-80% of BL cases, is the t(8;14)(q24;q32). Variant translocations t(2;8)(p12;q24) and t(8;22)(q24;q11) are observed in 10-15% (Kaiser-McCaw *et al*, 1977; Bernheim *et al*, 1981; Bertrand *et al*, 1981). These translocations result in the deregulation of *MYC* when its expression comes under the control of immunoglobulin gene (*Ig*) enhancers located on 14q, 2p or 22q (Bernheim *et al*, 1981; Dalla-Favera *et al*, 1982). *MYC* encodes a transcription factor that is essential for normal cell growth and proliferation, but when *MYC* becomes deregulated due to chromosomal translocations excessive proliferation occurs, thereby promoting oncogenic transformation (Boxer & Dang, 2001). A small portion of BL cases (7-19%) have been reported to lack detectable *MYC* rearrangements in both classical cytogenetic and molecular cytogenetic studies (Lones *et al*, 2004; Poirel *et al*, 2008; Haralambieva *et al*, 2004).

Approximately 60-90% of BLs contain secondary chromosomal abnormalities, with 30-50% of the tumors exhibiting complex karyotypes (Lones *et al*, 2004; Poirel *et al*, 2008; Onciu *et al*, 2006; Lai *et al*, 1989). Cytogenetic studies have consistently shown that when excluding the *MYC*-involved translocations, the most frequent aberrations include rearrangements of chromosomes 1, 6, 7, 12, 13 and 17, typically resulting in gain of 1q, 7q and 12q, and loss of 6q, 13q and 17p (Lones *et al*, 2004; Onciu *et al*, 2006; Lai *et al*, 1989; Kornblau *et al*, 1991; Barth *et al*, 2004). The accumulations of genetic aberrations affect disease biology, and often play a role in disease progression and impact prognosis (Harrison, 2001). A recent retrospective study by the FAB/LMB96 International Study Committee on abnormal karyotypic results from 238 children and adolescents with mature B-cell lymphoma (76% BL) reported that the most common clonal structural alterations observed in descending frequency were +1q, +7q, del(13q) and del(6q) (Poirel *et al*, 2008). Specifically, deletions of 13q and gain of 7q were each identified in 14% of the BL cases and were associated with a significant impact on prognosis with decreases in OS and EFS. In contrast, the most common secondary abnormality, +1q, occurred in 29% of the cases and had no detectable effect on clinical outcome (Poirel *et al*, 2008).

Given the prognostic value of specific cytogenetic changes in a variety of human malignancies, the aims of the current study were to determine the frequency of *MYC* rearrangements, 13q deletion and gain of 7 in Burkitt lymphoma by fluorescence in situ hybridization (FISH) and evaluate their potential prognostic significance in a cohort of 90 children and adolescents with intermediate and advanced BL treated on Children's Cancer Group study 5961. We found that deletions of specific regions of 13q have a significant impact on clinical outcome in patients with BL, whereas *MYC* rearrangement and gain of 7 did not affect outcome.

Patients, materials and methods

Patient Selection

The patient cohort for this study included children and adolescents (6 months to 19 years of age) registered on Children's Cancer Group Study CCG-5961 between May 15, 1996 and June 15, 2001. Patients registered on the CCG-5961 protocol were categorized into three groups according to risk and therapeutic protocols (Patte *et al*, 2001). Group A, comprising subjects with completely resected stage I or completely resected abdominal stage II lesions,

was excluded from this study. Group C includes stage IV subjects with CNS involvement and/or bone marrow involvement $\geq 25\%$ blasts and group B includes subjects not eligible for group A or C (Patte *et al*, 2001). Patients from groups B and C received two to three courses of reduction therapy with cyclophosphamide, vincristine and prednisone (COP) followed by induction therapy with additional multiagent intensive chemotherapy including doxorubicin and methotrexate (Cairo *et al*, 2007; Patte *et al*, 2007). The protocol was approved by all the local institutional review boards and written informed consent was obtained from all patients or their parent or legal guardian in accordance with the declaration of Helsinki.

Patient Demographics

The patient characteristics at diagnosis are summarized in Table I. The male:female ratio of the study group was 5:1. Seventy-six patients (84%) were children less than 15 years of age with the majority being 5 to 9 years of age (38%). Ethnicity was not included in patient demographics. When stratified according to risk, the majority of BL cases for this study fell within the group B regimen (78/90, 86%). Group C comprised 12 patients with advanced disease and bone marrow or CNS involvement. As of September 2008, 80 (89%) were alive at last contact and 10 (11%) were deceased, with nine of the ten deceased patients presenting at stage III or greater (Table II). The cause of death for 6/10 patients was disease progression prior to month 7, three died from therapy associated toxicities (1 hemorrhage, 2 infection) and one died from unrelated causes.

Tumour Samples

The primary biopsy samples were processed and evaluated at the individual registered institutions, including all immunophenotyping studies. A central pathology review, consisting of light microscopic examination of morphology and limited immunohistochemical analysis (CD20, CD79a, CD3, CD45RO and TdT), was performed and the BL diagnosis was established as defined according to the revised European-American classification (REAL) (Harris *et al*, 1994) which formed the basis of the current World Health Organization (WHO) classification (Leoncini *et al*, 2008). Only diagnostic specimens with a consensus classification of classical BL were considered eligible for this study. Routinely processed paraffin-embedded biopsy samples were selected for FISH analysis. Tumour samples included arose from various anatomic sites including lymph nodes, gastrointestinal system, bone marrow, and pleural and ascitic fluids, among others.

Fluorescence in situ Hybridization

FISH assays were performed on 4-5 μ thick unstained, paraffin-embedded tissue sections (USS) utilizing the Abbott/Vysis LSI[®] MYC Dual Color, Break Apart Rearrangement probe, and a cocktail of the Vysis LSI[®] D13S319 (13q14.3) SpectrumOrange[™], Vysis LSI[®] 13q34 SpectrumGreen[™] and the Vysis CEP[®] 7 (D7Z1) SpectrumAqua[™] probes mixed according to manufacturer's instructions (Abbott/Vysis, Abbott Park, IL). In order to prepare the slides for hybridization they were pretreated on the VP2000[™] Automated Tissue Processor (Abbott/Vysis, Abbott Park, IL) with standard protocols. Briefly, the slides were deparaffinized in Hemo-De, followed by dehydration in 95% ethanol. The slides were then incubated in 0.2N HCl, Vysis Pretreatment Buffer, protease solution, and post-fixation solution, followed by a final dehydration in an alcohol series of 75%, 85% and 95% ethanol. Following pretreatment, FISH was performed by co-denaturation of the paraffin tissue and probe mixture at 75-80° C for 3-5 minutes and incubated overnight at 37° C using the HYBrite system[™] (Abbott/Vysis, Abbott Park, IL). Adjustments were made to the temperature and duration of the denaturation in order to achieve optimal quality of the hybridization and preservation of the tissue. The following day the slides were briefly washed in 2 $\times$ sodium citrate (SSC)/.1%NP-40 at 74° C for 2 minutes and at ambient

temperature for an additional 2 minutes. The tissue was then counterstained with 4',6-Diamidino-2-phenylindole (DAPI) and coverslipped.

Hybridization signals were accessed in approximately 100 interphase nuclei on a fluorescent microscope equipped with appropriate filters and images acquired using the Cytovision Image Analysis System (Applied Imaging, Santa Clara, CA). Patient outcome results were blinded at the time of FISH analysis. Results for the *MYC* gene region were classified as normal or positive for rearrangement while probe signals for chromosome regions 13q14.3, 13q34 and 7 CEP were scored for variation from the normal diploid count (2 signals) and reported independently. In accordance with an institutional FISH database utilizing a minimum of 1,500 normal paraffin-embedded control tissues, normal cut-off values at 95% confidence intervals were applied (≤ 6 nuclei with split *MYC* signals, ≤ 30 with 0-1 or ≤ 12 nuclei with 3 or more CEP 7 signals, ≤ 30 with 0-1 or ≤ 10 with 3 or more 13q14.3 or 13q34 signals). Due to the sectioning of cells in paraffin-embedded tissues, truncation of probe signals will occur; thus a higher cut-off value is necessary to confidently access signal loss.

Statistical Analysis

The Kaplan-Meier method (Kaplan & Meier, 1958) was used to estimate the distributions of overall survival (OS) and event-free survival (EFS). The cumulative incidence estimator was used to estimate the rates of relapse and lymphoma specific mortality. OS times were calculated as the time from patient registration on protocol to the date of death or last follow-up contact. Patients who were alive at last contact were treated as censored for OS analysis. EFS was defined as the time from registration to either the date of disease progression or relapse, death, or last follow-up contact. Patients who were alive at last contact and who had not progressed were treated as censored for EFS analysis. Differences between survival curves were analyzed by the log-rank test. The Cox proportional hazards regression model was used to estimate hazard ratios (HR) and 95% confidence intervals of risk factors in univariate and multivariate analysis. All statistical tests were 2-sided and p-values less than 0.05 were considered to be statistically significant. The data analysis was conducted utilizing the SAS software (Version 9.2, SAS Institute Inc., Cary, NC, USA).

Results

Fluorescence in situ Hybridization

Molecular cytogenetic studies were performed on 90 BL cases utilizing DNA probes specific for 8q24/*MYC*, 13q14.3, 13q34 and centromere 7. Eighty-seven cases (97%) showed genetic alterations by FISH (Table III). Rearrangement of the *MYC* gene region was detected in 93% (84/90) of the cases (Figure 1a, 1d). Corresponding H&E stained tissue sections of the *MYC* negative cases were re-examined by an experienced haematopathologist with confirmation of the presence of adequate tumor tissue for FISH analysis, therefore, the *MYC* negative cases were included in the statistical analysis for survival. Gain of *MYC* (3-4 copies) and CEP 7 (centromere 7) was observed in one of six cases negative for rearrangement, while two cases contained abnormalities of chromosome 13. Three cases exhibited normal results for all FISH probes analyzed.

Deletions of 13q were present in 42% of the cases studied (Table III). Loss of signal for both D13S319 (13q14.3) and 13q34 was the most frequent FISH pattern observed (18/90), followed by loss of only D13S319 (13q14.3) in 13/90 cases and loss of only 13q34 in 7/90 cases. When assessed independently, loss of 13q14.3 occurred more frequently than loss of 13q34 (34% vs. 28%) (Figure 1b, 1e). For the purpose of survival analysis, two cases exhibiting 2 copies of D13S319 (13q14.3) and 3 copies of 13q34 were interpreted as relative loss of 13q14.3. Due to limited sample, only 68 of the original 90 pediatric BL cases could

be analyzed for copy number of chromosome 7 centromere (Table III). Gain of CEP 7 was noted in 7/68 (10%) (Figure 1c, 1f).

Karyotypic information was available on 18 cases (Table IV). There was complete concordance between the cytogenetic and FISH results for the presence of *MYC* translocations. Structural abnormalities resulting in loss of 13q material were detected in 8/18 (44%) cases by classical cytogenetics, while 14/18 (78%) cases showed 13q loss by FISH. This resulted in 57% concordance for the presence of 13q loss between the two methods; however, there was only 13% (1/8) concordance regarding the breakpoints. Gain of chromosome 7 was noted in one case by both conventional karyotyping and FISH.

Among deceased patients a deletion of 13q14.3 was observed in five of the six patients that died from disease progression. The remaining patient lacked a rearrangement of the *MYC* gene region as detected by FISH but showed gain of both *MYC* and chromosome 7 (Table II).

Survival Analysis of Cytogenetic Abnormalities

Univariate analysis of OS and EFS was performed for cytogenetic changes and the following other risk factors: age, gender, Murphy stage, LDH>2 times normal, bone marrow and/or CNS involvement. The follow-up time of surviving patients ranged from 1.1 to 7.4 years, with a median time of 4.6 years. The survival time for the deceased patients ranged from 1 to 47 months, with a median time of 4.28 months (Table II). The 5-year overall survival for all patients was 89%. The results of the univariate EFS and OS analysis for the different patient groups with 13q loss is presented in Figure 2. Patients exhibiting unspecified 13q abnormality had poorer OS relative to patients lacking a 13q abnormality ($p=0.011$); the estimated 5 year OS of patients with del(13) was 78% vs. 96% for patients without (Figure 3a). More specifically, loss of 13q14.3 was also associated with a significant decrease in OS (estimated 5 year OS, 77% vs. 95%, $p=0.012$) (Figure 3b). The results of the Cox regression model in multivariate analysis of OS are presented in Table V. Multivariate analysis revealed that the risk of death is significantly greater for patients with del(13) ($p = 0.03$, HR = 5.63, 95% CI: 1.17, 26.98) and del(13)(q14.3) ($p = 0.04$, HR = 4.17, 95% CI: 1.07, 16.22) relative to other patients after adjusting for LDH status and bone marrow and/or CNS involvement. Furthermore, there was a significant difference in the cumulative incidence of lymphoma specific death between patients with non-specific 13q deletions ($p=0.0114$) or patients with identifiable 13q14.3 deletions ($p=0.0124$) relative to patients lacking 13q deletion as accessed by FISH. Loss of 13q34 or loss of both 13q14.3 and 13q34 was not associated with poorer prognosis. Although del(13) and del(13)(q14.3) were associated with a poorer overall survival, they did not have a significant influence on EFS. Only bone marrow and/or CNS involvement was associated with a decrease in EFS on univariate analysis ($p = 0.0082$) while LDH level and bone marrow and/or CNS involvement were both associated with an inferior OS with $p = 0.0082$ and $p<0.001$ respectively. There was no statistically significant difference in EFS or OS for age, gender, Murphy stage, *MYC* disruption or gain of chromosome 7.

Discussion

The treatment of children and adolescents with Burkitt lymphoma has been systematically modified over the past few decades producing high cure rates using short duration but high intensity chemotherapeutic approaches that are associated with significant treatment-related toxicity. By taking into account various prognostic factors, it has been proposed that it may be possible to progressively reduce the duration and intensity of chemotherapy and treatment-related toxicities (Reiter *et al*, 1999; Gerrard *et al*, 2008; Patte *et al*, 2007). This is the first large molecular cytogenetic study conducted on BL arising in children and

adolescents specifically aimed at detecting chromosomal aberrations recently reported to provide prognostic information among patients within this group (Lones *et al*, 2004; Poirel *et al*, 2008; Onciu *et al*, 2006). Classical cytogenetics is dependent upon the availability of fresh tissue and the presence of metaphase cells, which is often limited by resolution and suboptimal morphology, for accurate determination of numerical and structural chromosome anomalies. The advent of molecular cytogenetic techniques, in particular FISH, has circumvented these limitations and has provided a rapid and straightforward procedure for the detection of specific chromosomal abnormalities.

Since their initial characterization in the late 1970's, rearrangements involving the *MYC* gene region have been considered the cytogenetic and molecular hallmark of BL (Kaiser-McCaw *et al*, 1977). Although the WHO Classification states that all BL cases have a translocation of *MYC* to either the *Ig* heavy chain locus or one of two *Ig* light chain loci (Leoncini *et al*, 2008), there is accumulating evidence that a small portion may lack identifiable *MYC* rearrangements (Haralambieva *et al*, 2004; Hummel *et al*, 2006). In the present study, 7% of the cases were negative for a *MYC* rearrangement by FISH analysis, despite confirmation of tumour by morphology. This is comparable to previous classical cytogenetic and molecular cytogenetic studies reporting 7-19% of BL cases without detectable *MYC* translocations (Lones *et al*, 2004; Poirel *et al*, 2008; Haralambieva *et al*, 2004). The detection of chromosomal abnormalities other than *MYC* rearrangement in 3/6 cases provided additional evidence for the presence of lymphoma. Consistent with previous reports (Poirel *et al*, 2008), *MYC* status was not associated with an inferior prognosis in this study, but accurate prognostic evaluation may be hindered by the limited number of *MYC* negative cases.

The failure to detect *MYC* rearrangement may be attributed to the FISH probe used. There is considerable variability in the *MYC* rearrangements described in BL, including translocations several kb upstream and downstream from *MYC* and insertion of *MYC* into the *IGH* region (Haralambieva *et al*, 2004; Zeidler *et al*, 1994; Joos *et al*, 1992). Although the *MYC* probe set utilized in this study was designed to allow for detection of breakpoints over a large region including the classic t(8;14), as well as the variant t(2;8) and t(8;22), a limited number may not be detected (Figure 1a). Interestingly, a gene expression profiling study by Hummel *et al*. identified a subset of mature aggressive B-cell lymphomas with the molecular signature of BL but without *MYC* rearrangement, thus providing further evidence for the existence of true BL cases lacking *MYC* rearrangements (Hummel *et al*, 2006). Alternatively, the microRNA (miRNA), hsa-mir-34b, might be involved in *MYC* deregulation. Leucci *et al*. demonstrated that *MYC* was deregulated in BL cases lacking *MYC* rearrangements in association with hsa-mir-34b down-regulation (Leucci *et al*, 2008).

Chromosome 13q loss, predominantly involving the 13q14.3 region, was observed in 42% of the BL cases from this study and was associated with an inferior overall survival. Eighty percent of the patients who died exhibited abnormalities of 13q, 70% showed loss of 13q14.3 and 60% died of disease progression. A recent FAB/LMB 96 study of cytogenetic abnormalities in mature B-cell NHL reported that patients with del(13q) had a significantly inferior response to COP reduction therapy (Poirel *et al*, 2008). They also reported that the most commonly deleted region on chromosome 13 involved band q34 (82%) (Poirel *et al*, 2008). In 87% (7/8) of the cases containing 13q cytogenetically visible abnormalities the breakpoints could be further refined based on the information provided by the loci specific probes, thus providing a possible explanation for the discrepancy in the most commonly deleted 13q region between the current study and the previous FAB/LMB96 study. Presumably these 13q losses are associated with tumour suppressor gene loss.

Loss of 13q has been reported as the second most common recurring abnormality (16-27%) in prior cytogenetic studies of BL (Lones *et al*, 2004; Onciu *et al*, 2006; Berger *et al*, 1989).

This is in contrast to the 42% abnormality rate for del(13q) detected in the current study (Table III). When comparing the cytogenetic and the FISH data it is apparent that 43% (6/14) of the cases with abnormalities of 13q were not detected by classical cytogenetics (Table IV), possibly due to either submicroscopic deletions or the presence of additional non-proliferating subclones. Deletions of 13q not readily identified by conventional cytogenetic analysis have been reported in several other B-lymphoid malignancies, such as chronic lymphocytic leukemia (CLL) and multiple myeloma (MM) (Aoun *et al*, 2004; Chang *et al*, 2004). Notably, 13q loss is an important indicator of inferior prognosis in MM (Kaufmann *et al*, 2003; Zojer *et al*, 2000). In the current study, a poorer OS was observed for the combined patient group with non-specific 13q loss and for the patient group exhibiting specifically 13q14.3 loss with or without deletion of 13q34, but not for the group exhibiting 13q34 loss with or without deletion of 13q14.3. However, analysis of the 13 patients with deletion of 13q14.3 and normal 13q34 showed no association with inferior OS or EFS. This may suggest that although 13q14.3 loss appears to play a role in disease progression, there may be additional regions of interest on chromosome 13 outside of q34. This data also suggests that there was not an association between 13q loss and patients who have relapsed or not achieved a remission with initial therapy and then subsequently salvaged with another treatment strategy. Although deletions of 13q discernable by classical cytogenetics have been shown to be associated with a significant decrease in OS and EFS previously (Poirel *et al*, 2008), the critical regions of loss have not defined.

Attempts to define a minimal region of loss on 13q in BL, and thus identify specific genes that may be used as informative prognostic indicators, have been difficult due to the limited data available and to the large variability of the deleted regions ranging from 13q14 to 13q34 (Lones *et al*, 2004; Onciu *et al*, 2006; Berger *et al*, 1989). These regions include many known genes encoding for proteins that function as transcription factors, cell cycle regulators and oncogenes/tumor suppressors (Dessen *et al*, 2002). Of particular interest are two tumor suppressor genes known to lie within the region covered by the 13q14.3 FISH probe utilized in this study, *DLEU1* and *DLEU2* (deleted in lymphocytic leukemia 1 and 2) (Figure 1a) (Abbott Laboratories 2008; Liu *et al*, 1997). *DLEU1* and *DLEU2* have been defined as *MYC* target genes (Dave *et al*, 2006; Li *et al*, 2003). Although *DLEU1* is a non-protein coding gene whose function remains ill-defined, it has been identified as a signature upregulated gene within the Burkitt classifier used to distinguish BL from diffuse large B-cell lymphoma (Dave *et al*, 2006). The *DLEU2* tumor suppressor gene encodes two miRNAs, miR-15a and miR-16, thought to play an important role in B-cell neoplasms, particularly CLL (Calin *et al*, 2002). miR-15a and miR-16 have been shown to induce apoptosis by negatively regulating the anti-apoptotic gene *BCL2* (B cell lymphoma 2) (Cimmino *et al*, 2005). No specific genetic alterations other than loss have been associated with these genes.

Several studies have described trisomy 7 or gain of 7q as recurrent secondary abnormalities of BL, with reported frequencies ranging from 11-15% (Poirel *et al*, 2008; Onciu *et al*, 2006; Kornblau *et al*, 1991; Garcia *et al*, 2003; Salaverria *et al*, 2008) and in some instances it has been associated with inferior clinical outcome (Poirel *et al*, 2008; Garcia *et al*, 2003). One study noting a decrease in EFS in the presence of +7q, reported that the minimal region of gain was restricted to 7q21q22 with only about half of the cases involving a whole chromosome gain (Poirel *et al*, 2008). Chromosome 7 gain was present in 10% of the cases from the current study but an association with an inferior event-free or overall survival was not identified; however, due to the unavailability of commercial probes specific for this region, the data from the current study was limited by the use of a centromeric probe for chromosome 7. It is possible that an inferior outcome was not detected with the gain of chromosome 7 due to the inaccurate assessment for the critical region or the low number of cases with +7 present in this patient group. Although trisomy 7 is not an uncommon finding

in non-neoplastic lesions as well as apparently normal tissue (Bardi *et al*, 1992; Heim *et al*, 1989; Johansson *et al*, 1993), it has been reported that trisomy 7 may be related to tumour progression in NHL (Knutsen, 1998). The frequency of 7q gain in BL may provide suggestive evidence for a contribution to tumour cell growth due to increased gene dosage.

In summary, these data provide a comprehensive analysis of 13q loss by FISH in a large series of uniformly treated children and adolescents with BL. These findings indicate that loss of 13q occurs at frequencies much higher than previously reported in classical cytogenetic studies and that FISH analysis is necessary to more accurately assess the presence of these abnormalities and to correctly identify the regions of loss. The association of 13q14.3 loss with inferior overall survival may provide a rational basis for further refining the risk stratification of BL patients so that therapy can be appropriately modified. In addition, a decreased OS in the group with all non-specific del(13q) cases, suggests that additional 13q regions may also be of importance in tumour progression indicating that more complete analysis of 13q loss may be necessary. The data provided here suggest that specific genes may impact the biological behavior of BL and could provide potential new therapeutic targets.

Acknowledgments

The authors would like to acknowledge the Children's Oncology Group for the tissue samples and all the CCG/COG committee members especially Richard Spoto PhD, study statistician and investigators who participated in the trial. This work was supported in part by grants from the Divisions of Cancer Treatment, National Cancer Institute, National Institute of Health and Department of Health and Human Services (COG). They would also like to acknowledge the Human Genetics Laboratory at UNMC for funding the FISH analysis. The authors would also like to thank the members of the DSMC, Professor Michael Link, Alfred Reiter, David Harrington and Robert Souhami for their diligent oversight of this study.

Supported by grants from Chair's Grant U10 CA98543 and the Statistics and Data Center Grant U10 CA98413 of the Children's Oncology Group from the Divisions of Cancer Treatment, National Cancer Institute, National Institute of Health and Department of Health and Human Services. The authors have no other financial conflict of interest to disclose.

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Figure 1.

FISH analysis for the detection of *MYC* rearrangement, deletion of 13q and gain of chromosome 7. (A) Schematic display of Vysis LSI[®] MYC Dual Color, Break Apart probe, centromeric portion labeled in SpectrumOrange[™] and telomeric portion labeled in SpectrumGreen[™]. (B) Schematic display of Vysis LSI[®] D13S319 (13q14.3) probe labeled in SpectrumOrange[™], Vysis LSI[®] 13q34 probe labeled in SpectrumGreen[™]. (C) Schematic display of Vysis CEP[®] 7 (D7Z1) probe labeled in SpectrumAqua[™]. (D) Interphase nuclei demonstrating rearrangement of the MYC dual color, break apart probe as indicated by one colocalized (fused) orange and green signal, 1 separate orange signal and 1 separate green signal. (E) Interphase nuclei demonstrating deletion of LSI 13q14.3 and normal copy number for LSI 13q34 as indicated by 1 orange signal and 2 green signals. (F) Interphase nuclei demonstrating gain of CEP 7 and normal copy number of LSI 13q14.3 and 13q34 as indicated by 3 aqua signals, 2 orange signals and 2 green signals. (D-F shown at x100 original magnification)

Figure 2.

Event-free survival (EFS) and overall survival (OS) for various patient groups with 13q loss. P-values for OS among patient groups “unspecified del(13)” and “del(13)(q14)” fall below 0.05, demonstrating an association with these groups and decreased OS.

Figure 3. Univariate analysis of 5-year OS in 90 children and adolescents with intermediate and high-risk Burkitt lymphoma. (A) OS with and without deletion of chromosome 13. (B) OS with and without deletion of 13q14.3.

Table I

Patient characteristics.

	Total: 90 (%)
Gender	
Male	75 (83.33)
Female	15 (16.67)
Age Groups	
0 to 4 years	14 (15.56)
5 to 9 years	34 (37.78)
10 to 14 years	28 (31.11)
15 to 19 years	14 (15.56)
Stage (Risk group)	
Stage I: NR (B)	6 (6.67)
Stage II: ANR (B)	21 (23.33)
Stage III: (B)	48 (53.33)
Stage IV: M-C- (B)	3 (3.33)
Stage IV: M-C+ (C)	3 (3.33)
LAL: C- (C)	5 (5.56)
LAL: C+ (C)	4 (4.44)
Alive	
Yes	80 (88.88)
No	10 (11.11)

NR, non-resected; B, group B regiment; ANR, abdominal non-resected; M-C-, bone marrow and CNS negative; M-C+, bone marrow negative and CNS positive; C, group C regiment; LAL: C-, bone marrow with $\geq 25\%$ blasts and CNS negative; LAL: C+, bone marrow with $\geq 25\%$ blasts and CNS positive.

Clinical characteristics and FISH results of deceased patients.

Table II

Patient	Survival time in months	Stage at diagnosis	Cause of death	MYC	13q14.3	13q34	CEP 7
1	47	LAL C+ (C)	Other	Split	Deleted	Deleted	Gain
2	1	LAL C- (C)	Lymphoma	Split	Deleted	Deleted	Normal
3	2.6	LAL C+ (C)	Hemorrhage	Split	Deleted	Normal	ND
4	7.3	Stage II: ANR (B)	Infection	Split	Normal	Deleted	ND
5	5.5	LAL C+ (C)	Lymphoma	Split	Deleted	Deleted	Normal
6	4.25	Stage III: (B)	Lymphoma	Gain	Normal	Normal	Gain
7	12.5	Stage III: (B)	Infection	Split	Normal	Normal	Normal
8	4.3	Stage III: (B)	Lymphoma	Split	Deleted	Normal	Normal
9	3.3	LAL C- (C)	Lymphoma	Split	Deleted	Normal	ND
10	6.75	Stage III: (B)	Lymphoma	Split	Deleted	Normal	ND

LAL: C+, bone marrow with $\geq 25\%$ blasts and CNS positive; C, group C regimint; LAL: C-, bone marrow with $\geq 25\%$ blasts and CNS negative; ANR, abdominal non-resected; B, group B regimint; ND, not determined.

Table III

Fluorescence in situ hybridization results.

Probe region	Total # of cases	Normal (%)	Split (%)	Deleted (%)	Gain (%)
8q24/MYC	90	5 (5.55)	84 (93.33)	-	1 (1.11)
13q	90	52 (57.78)	-	38 (42.22)	-
13q14.3 and 13q34		72 (80.0)	-	18 (20.0)	-
13q14.3 (with normal 13q34)		77 (85.56)	-	13 (14.44)	-
13q34 (with normal 13q14.3)		83 (92.22)	-	7 (7.78)	-
13q14.3 (with or without normal 13q34)		59 (65.56)	-	31 (34.44)	-
13q34 (with or without normal 13q14.3)		65 (72.22)	-	25 (27.78)	-
7 CEP	68	61 (89.91)	-	-	7 (10.29)

Table IV

Comparison of cytogenetic and FISH results from 18 cases.

Case	Cytogenetics	FISH			
		MYC	D13S319 (13q14.3)	13q34	CEP 7
1	47,XX,t(8;14)(q21;q32),t(14;15)(q32;q21),+i(18)(p10)c[3]/47, idem,del(13)(14)[3]/47,XX,i(18)(p10)c[16]	Positive	Deleted	Negative	ND
2	46,XX,t(8;14)(q24;q32)[11]	Positive	Negative	Negative	Normal
3	46,XY,t(8;14)(q24;q32)[6]/46,XY[8]	Positive	Deleted	Deleted	ND
4	46,XY,t(8;14)(q24;q32)[2]/46,XY,dup(1)(q21q32),t(8;14)(q24;q32), der(13)t(7;13)(q11.2;q22)[3]/46,XY[15]	Positive	Deleted	Deleted	Normal
5	46,XY,t(8;14)(q24;q32)[11]/87,XXY,-Y,-2,-3,t(8;14)(q24;q32)x2,-der(8)t(8;14),-14[4]	Positive	Deleted	Negative	ND
6	46,XY,dup(1)(q12q23),t(8;22)(q24;q11)	Positive	Deleted	Deleted	ND
7	46,XX,t(8;14)(q24;q32)[20]	Positive	Deleted	Deleted	ND
8	46,XY,t(8;14)(q24;q32),del(13)(q14),der(13;17)(q10;q10),+mar [10]/46,XY[9]	Positive	Deleted	Deleted	Normal
9	48,XY,+7,t(8;14)(q24;q32),+19 [20]	Positive	Negative	Negative	Gain
10	46,XX,t(8;14)(q24;q32)[1]/46,XX [2]	Positive	Negative	Negative	Normal
11	46,XY,add(2)(p23),der(9)t(2;9)(p23;p21),add(11)(q23),der(12) t(9;12)(p21;q21),del(13)(q21),del(22)(q13)[18]/47,sl,del(1)(p13), +mar[2]	Negative	Deleted	Negative	ND
12	46,XY,t(8;14)(q24;q32)	Positive	Negative	Negative	Normal
13	46,XX,del(1)(q21),t(2;3)(p32;p21),add(6)(q2;5),t(8;14)(q24;q32), inv(10)(p11.2q32),der(13)add(13)(p11.2)t(1;13)(q24;q12) [6]/46,sl,+der(1)t(1;3)(q25;q12),-del(1)[16]	Positive	Deleted	Negative	ND
14	49,XY,t(8;14)(q24;q32),+12,add(13)(q32),+add(18)(q22),+10[16]/ 49,sl,t(1;17)(q32;p11.2)[4]	Positive	Deleted	Deleted	ND
15	46,XX,t(8;14)(q24;q32)[17]/46,sl,del(17)(p12)[3]	Positive	Deleted	Deleted	Normal
16	46,XX,t(8;14)(q24;q32)[20]	Positive	Negative	Deleted	Normal
17	46,XY,t(8;14)(q24;q32)[8]/46,sl,add(13)(q22)[?]/47,sl,+1[3]/48,sl, +1,+8[2]	Positive	Deleted	Negative	ND
18	50,XY,t(8;14)(q24;q32),+add(17)(q25),+mar,+t(cp10)/49,XY, t(8;14)(q24;q32),add(13)(q32),+add(17)(q25),+mar[8]	Positive	Deleted	Deleted	ND

ND, not determined.

Table V

Multivariate model for OS in patient with unspecified del(13)

Characteristic	Risk Factor	Parameter Estimate	P-value	Hazard Ratio	95% CI
Unspecified del(13)	Present vs. Absent	1.728	0.03	5.63	1.17 26.98
LDH > 2 x normal	Yes vs. No	1.497	0.18	4.47	0.51 39.47
BM+/CNS+	BM+/CNS+ vs. other	1.756	0.009	5.79	1.55 21.63
Multivariate model for OS in patient with del(13)(q14)					
Characteristic	Risk Factor	Parameter Estimate	P-value	Hazard Ratio	95% CI
del(13)(q14)	Present vs. Absent	1.427	0.04	4.17	1.07 16.22
LDH > 2 x normal	Yes vs. No	1.658	0.13	5.25	0.60 46.00
BM+/CNS+	BM+/CNS+ vs. other	1.529	0.02	4.62	1.25 17.06

Unspecified del(13) and del(13)(q14) are not included in the same model because they are closely associated; all patients with del(13)(q14) (n=31) are included in the unspecified del(13) group.
LDH, lactate dehydrogenase; BM+/CNS+, bone marrow with > 25% blasts and central nervous system involvement.