Genomic signatures in pediatric advanced stage Burkitt lymphoma/leukemia in Chinese population by next generation sequencing

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Abstract
Background: Burkitt lymphoma/leukemia (BL/BAL) is the most common lymphoma in children and sporadic subtype is dominant in Chinese population. MYC gene translocation is essential for sporadic BL/BAL (sBL/BAL), whereas other gene mutations also play important roles in the development of sBL/BAL. Methods: The clinical data of ten Chinese children with sBL/BAL were collected; next generation sequencing of tumor tissues were detected. BL and diffused large B cell lymphoma (DLBCL) database was also collected and bioinformatics analyzed was done. Results: Nine male and one female were enrolled in the study, including six BL patients (stage III) and four BAL patients (stage IV). Average age at diagnosis was 100.10±13.39mon; overexpression of CD20 was detectable, MYC rearrangement were confirmed. The patients received combination treatment of chemotherapy and Rituximab, complete remission was achieved and all the patients survived. Germline causal gene mutations were detected in four (40%) patients by whole exome sequencing (WES); ID3, BRCA2, ARID1A and SMARCA4 mutations, except MYC mutation, were the most common somatic mutations. The gene functions in pathopoiesis were differ from BL to DLBCL in dataset. These identified mutated genes were enriched and connected by GO or KEGG pathway, it seemed that PI3K-Akt signaling pathway or EGFR-TKI resistance pathway play important roles in the pathopoiesis of sBL/BAL. Conclusion: sBL/BAL is a highly aggressive but curable lymphoma; the pathophysiolopoesis is still in research. The molecular hallmark of sBL/BAL is MYC translocation, whereas additional chromosomal abnormalities and gene mutations also occur and play roles in the progression of the disease. PI3K-Akt signaling pathway seems play important roles in the pathopoiesis of sBL/BAL,
and sBL/BAL may be inhibited by such signaling pathway; EGFR-TKI resistance pathway was also analyzed in sBL/BAL patients, it reveals that EGFR-TKI treatment is invalid. Further research is needed for the hypothesis and possible mechanisms.

**Key words:** Burkitt lymphoma; children; next generation sequencing; whole exome sequencing.

**Introduction**

Burkitt lymphoma/leukemia (BL/BAL) is most common subtype of non-Hodgkin lymphoma (NHL) in children and adolescent, it occurs 30-50% of pediatric NHL populations; there are three reported variants of BL/BAL: endemic, sporadic and immunodeficiency-related BL/BAL[1]. Sporadic BL/BAL (sBL/BAL) is familiar throughout the world and it is common in Chinese race rather than endemic or immunodeficiency-associated BL/BAL [2].

Murphy staging system or revised international pediatric non-Hodgkin lymphoma staging system (IPNHLSS) has been proposed for pediatric BL cases; localized-stages (stage I or II) or advanced-stages (stage III or IV) are classified respectively [3]. With intensive combination chemotherapy, the prognosis of sBL/BAL in children and adolescents had been improved dramatically. Currently, 5-year event-free survival (EFS) of local or advanced stages of BL/BAL had reached to -100% or 85–90% respectively [1, 2].

It is well known that t(8;14)(q24;q32) or its variants played key role in BL/BAL, translocation of MYC gene, which is located at band 8q24, is detectable in over 95% cases; Epstein–Barr virus (EBV) is also common in BL/BAL. The MYC translocation is the essential driver for overexpression of MYC gene, activation of MYC gene leads to cell cycle progression, inhibition of differentiation and effects promoting cell proliferation and/ or genomic instability [4]. Additional chromosomal abnormalities, recurrent transcript and/or gene mutations were also detected in BL/BAL patients and played their roles in the arising, progression and aggression of disease [5]. For instance, somatic mutation of TCF3 can activate the PI3K/MAPK/MTOR pathway, in part by increasing a tonic form of B cell receptor signaling; patients with germline mutation in SH2 domain protein 1A gene (SH2D1A) suffered from increased risk of BL/BAL [6].
Next-generation sequencing (NGS) studies had provided valuable insight into the landscape of genomic alterations in malignancies by using Whole exome sequencing (WES) and/ or RNA sequencing (RNAseq), it is helpful for researcher to elaborate the arising, pathogenesis and mechanism of such diseases [7], Bioinformatics analysis has been widely used in cancer research. WES sequencing or mRNAseq for sBL/BAL in Chinese children has not been demonstrated. In this study, we detected WES and/ or RNAseq in ten sBL/BAL patients, investigated the role of individual ID family members in BL/BAL and diffused large B cell lymphoma (DLBCL) using large databases and try to reveal the signaling pathway in pathogenesis and its relationship between clinical character and gene mutations.

Patients and Methods

Patients

Ten patients with newly diagnosed sBL/BAL at Children Hospital of Chongqing Medical University (CHCMU) between February 2018 and October 2019 were enrolled in the study. The diagnosis is in accordance with the World Health Organization criteria of 2016, and patients were staged with revised IPNHLLS [3]. Patients who were ≥18yr at diagnosis, diagnosed with Burkitt-like lymphoma with 11q aberration, secondary lymphoma, or had human immunodeficiency virus infection were excluded; patients who were classified with local stages (stage I or stage II), who had received chemotherapy before hospitalized, were also excluded from the study. The patients received chemotherapy in accordance to modified non-Hodgkin Lymphoma 1995, Berlin-Frankfurt-Münster, (BFM-95) protocol [8, 9]. Intrathecal injection (iT) was administrated as protocol required, cranial radiotherapy was carried out for these patients with central nervous system (CNS) involvement. The detail of risk group, course of treatment and drugs dosage for modified BFM-95 protocol were listed in Supplementary (S1-S4).

The Ethics Administration Office of CHCMU granted ethic approval for the research, informed consents were obtained from the patients or their guardians. Clinical data, laboratory findings and prognosis data of enrolled patients were collected and analyzed retrospectively from the medical record system.
Pathologic diagnosis

Pathologic diagnosis of BL patients were confirmed by lymph node (LN) biopsy. Immunohistochemical (IHC) staining was detected, catalog of IHC included: MYC, Ki-67, CD20, BCL-2, BCL-6, PAX-5, CD10, CD7, CD3, MUM-1, et al; fluorescence in situ hybridization (FISH) of MYC, MLL, BCL-2, BCL-6 and EBER were also detected [10].

Diagnosis and classifications of BAL patients were done by bone marrow (BM) samples. BM samples were determined by morphology of FAB typing, flow cytometry (FCM), cytogenetic analysis of chromosomal karyotype, FISH of MYC, ETV6-RUNX1, MLL, BCR-ABL1, TCF3-PBX1 and PDGFRB; 43 fusion genes which were detected by multiplex RT-PCR as literature report [11].

DNA, RNA isolation and sequencing

Tumor DNA samples of BL or BAL patients were obtained from formalin-fixed specimen or BM samples at diagnosis; germline samples were collected from oral mucosa of patients and their parents’ peripheral blood (PB). Genomic DNA was extracted using QIAmp DNA Minikit (QIAGEN, China). Genomic DNA was enriched and sequence acquisition was carried out (Agilent SureSelect Human All Exon V6); PCR amplifications of whole exome were sequenced (Illumina hiseq PE 150bp).

BM samples at diagnosis were collected from BAL patients, total RNA was extracted using QIAamp RNA Blood Mini Kit (Qiagen, Cat.52304). Enriched and captured mRNA library was constructed and amplified by using KAPA mRNA HyperPrep Kit (KAPA/Roche, Cat.KK8581). PCR amplifications of mRNAseq were sequenced using PE150 (Illumina hiseq ×10).

The original whole exome sequencing (WES) data were read using Illumina pipeline Software (version 1.3.4) and data were conducted referred to database (dbSNP, 1000 Genomes Project, ClinVar, ESP6500, ExAc, Ensembl, HGMD, UCSC, et al). Mutated genotypes were determined using software of GATK, LRT, Mutation Taster and SamTools.

Discovered variants were divided into the following four categories according to
literature reports [12] and software analysis: 1) Pathogenic genotypes that were confirmed by literature reports; 2) Likely pathogenic genotypes that were reported by literatures and, or affected protein by function predicting. 3) Indefinite variants; 4) Single nucleotide polymorphism (SNP) or single nucleotide variants (SNV). Pathogenic genotypes and likely pathogenic genotypes were recorded as causal gene mutations, causal gene mutations of tumor samples were confirmed by Sanger sequencing. Samples of control group were cross-checked and also detected by Sanger sequencing, somatic or germline causal gene mutations were identified.

**PPI network construction and GO, KEGG pathway enrichment analysis**

The detectable mutations by WES sequencing were collected, and R-package wordcloud2 was used to visualize the frequency of mutations in these cases. We used STRING (STRING, [http://string-db.org](http://string-db.org), RRID: SCR_005223) to find protein to protein interaction (PPI) and visualize the interaction using Cytoscape (version3.7.1). The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using R package clusterProfiler and Biological Networks Gene Ontology tool (BiNGO, RRID:SCR_005736).

**Identification of DEGs between BL and DLBCL dataset**

The gene chip dataset GSE4475, GSE10172, GSE43677, GSE48435 was downloaded from the Gene Expression Omnibus (GEO) database ([https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/)). We select BL and DLBCL dataset for downstream analysis. After quality control (QC), we got 41 BL samples and 178 DLBCL samples. Differential expression analysis was used R package limma. Database of essential genes (DEGs) were screened at a statistical significance Benjamini and Hochberg false discovery rate-adjusted \( p \)-value cutoff of 0.05 and an absolute value of fold change greater than 1. Volcano plot show DEGs using R language ggplot2.

**WGCNA analysis of the BL and DLBCL dataset**

We used R package weighted gene co-expression network analysis (WGCNA) to construct coexpression modules. 219 samples were used to calculate the Pearson’s Correlation Matrices. A power of 6 was selected. A unsigned hybrid co-expression
network was then calculated using standard settings. We select 5000 genes to construct topological heatmap. We did a *Pearson* correlation between the module eigengenes and the traits data to identify module-trait relationships. At last, we select blue module (related to BL) and red module (related to DLBCL) genes to construct gene regulatory network and did the GO enrichment analysis.

**Statistical Analysis**

Event was defined as each of the following situation: refractory or relapsed disease, disease progress, death or diagnosis of secondary malignancy. With following up to April of 2020, data of clinical features, laboratory findings, WES sequencing or RNAseq, treatment responses, treatment related mortality (TRM) and event free survival (EFS) rate of the patients were collected and analyzed.

EFS was calculated from date of diagnosis to last follow-up, loss of following-up or to the first event. SPSS 19.0 (IBM Corp., Armonk, NY) software was applied for statistical analysis. Survival curve were calculated according to Kaplan-Meier test. Proportional differences between patient groups were analyzed by *Pearson* chi-squared ($\chi^2$) tests or Fisher's exact tests. $P$ value <0.05 was regarded as statistical different.

**Results**

**Clinical, laboratory and pathologic characteristics**

Nine male and one female were enrolled in the study, the six BL patients were classified with stage III and the four BAL patients were regarded as stage IV. Age at diagnosis was 34-172m (median value: 105m; average value: 100.10±13.39m), two BL patients and one BAL patient presented with symptom B (fever), malignancy history was not found in their families. Average values of white blood cell (WBC), platelet (PLT), hemoglobin (Hb) and lactate dehydrogenase (LDH) was 13.90±6.92×10⁹/L, 315.20±54.39×10⁹/L, 101.90±6.94g/L and 2896.21±1328.56U/L respectively. Organ involvements were inspected by physical exam, ultrasonography, computed tomography (CT) or positron emission tomography (PET-CT), CNS involvement and bulky disease were found in one and six patients respectively.

The four BAL patients were diagnosed with ALL-L₃ by FAB morphology;
matured B-ALL phenotypes were performed and overexpression of CD20 was detectable, restrict expression of Kappa or Lambda chains were detected in two and two patients respectively; t(8;14) and/or MYC rearrangement were confirmed by chromosomal karyotype and/or FISH; common fusion genes were undetectable. The six BL patients were confirmed by LN biopsy. Overexpression of CD20, Ki-67 and MYC were detected by IHC staining; FISH of MYC and EBER was positive, FISH of BCL-2, BCL-6 or MLL was negative.

**Treatment and prognosis**

Six BL patients (five patients were classified as R3 group; one patient was regarded as R2 group) and four BAL (R4 group) patients, received chemotherapy in accordance to modified BFM-95 protocol. Treatment effects were evaluated after one or two courses of chemotherapy for BAL or BL patients respectively, all of them achieved complete remission (CR). Chemotherapy were finished in these patients in 5-6 mon. After that, the patient with CNS involvement received cranial radiotherapy (total dosage of 18Gy, divided into 10 times). With following-up to May of 2020, the 10 patients were alive without events, treat related mortality (TRM) was 0% and EFS rate was 100%.

**Results of NGS sequencing**

WES sequencing were supplied for the ten patients, germline causal gene mutations were detected in four (40%) patients; several somatic causal gene mutations were identified in the ten patients.

The data obtained by WES sequencing were analyzed using R-package wordcloud2 to visualize the frequency of candidate gene mutations, PPI network, GO enrichment and KEGG pathway analysis in these patients. (1) Identified gene mutations were showed by tag cloud using word size according to the gene frequency (Fig-1a), it demonstrated that ID3, BRCA2, ARID1A and SMARCA4 mutations, except MYC mutation, were the most common mutations; the identified gene mutations were also accumulated by PPI network (Fig-1b), it showed that proteins which were modified by MYC, HRAS, TP53 and NOTCH1 genes were the key proteins. The figure indicates that these identified gene mutations play important roles in the pathopoiesis
of BL/BAL. (2) Identified genes were enriched by GO analysis (Fig-2a). P value was defined as 0.05, the top gene functions in pathopoiesis were: leukocyte differentiation and regulation of hemopoiesis in biological process (BP), nuclear chromosomal part and chromatin in cellular component (CC), chromatin binding and transcription coregulator activity in molecular function (MF); these genes were also enriched and connected by KEGG pathway (Fig-2b), it seemed that PI3K-Akt signaling pathway was the key role in the pathopoiesis of BL/BAL.

RNAseq was supplied and gene transcripts were analyzed for the four MAL patients, the results were listed in Supplements.

Identification of DEGs and WGCNA analysis

For further understanding the pathophysiolopoiesis of BL/BAL, up-regulated genes were selected in BL and DLBCL dataset, we identified DEGs and get WGCNA analysis of between BL and DLBCL dataset, genes expression analysis for BL and DLBCL microarray data showed different by volcano plot (Fig-3); up-regulated genes in BL or DLBCL dataset was enriched by GO analysis and KEGG pathway respectively (Fig-4 and Fig-5), P value was also defined as 0.05, the gene functions in pathopoiesis were completely differ from BL to DLBCL in dataset.

The Topological Overlap Matrix (TOM) of co-expressed genes in different modules in the top 5000 genes was showed by Heatmap (Fig-6), eigengene adjacency heatmap for different modules was also presented. Module and trait relationships. The co-expression network of the significant genes related to BL (Fig-7a) or DLBCL (Fig-7b) was presented; GO enrichment of the significant genes related to BL (Fig-8a) and DLBCL (Fig-8b) were also demonstrated. It also presented with different co-expression networks or GO enrichment genes.

Discussion

BL/BAL is an aggressive non-Hodgkin lymphoma which was derived from germinal center B cells, and BAL is regarded as leukemic phrase of BL [1]. The peak age of sBL/BAL was 11yr and male is common than female, the enrolled ten patients included nine male and one female, the average age of these patients was 100.10±13.39m, clinical data in the study is similar to literature review [1-2, 10].
The prognosis of sBL/BAL remains poor in past decades, with short, intensive chemotherapy, the survival rate had been improved steadily. Overexpression of CD20, restrict expression of Kamppa or Lambda chain are remarkable in BL/BAL, with combination treatment of intensive chemotherapy and CD20 monoclonal antibody (Rituximab), the prognosis of pediatric sBL/BAL had exceed to 90%, EFS of the ten patients in the study was 100%, the treatment and diagnosis for these patients was alike with literatures [13-14].

The molecular hallmark of BL/BAL is translocation of the oncogenic MYC, similar translocations are also expressed in other NHL [10]. Although translocation of oncogenic MYC is detectable in these subtypes of NHL, clinical manifestation and prognosis remained diversity, diversity of sBL/BAL is also existed in children and adult [1, 4, 15]. It suggest that pediatric sBL/BAL can be distinguished from others by gene expression profiling, and differentiation of gene expression profiling potentially reflect distinct pathogenetic mechanisms.

Dataset of BL and DLBCL were collected and differential expression analysis was analyzed, statistical significance were presented; it reveal different gene expression profiling between BL and DLBCL, and it also indicate that the pathophysioloiesis between BL and DLBCL was distinct.

Data of WES sequencing in the ten pediatric sBL/BAL of Chinese populations were achieved and analyzed. ID3, BRCA2, ARID1A and SMARCA4 mutations, except MYC mutation, were common (Fig-1). By literature review[10,15-18], NGS sequencing analysis has revealed the importance of the B-cell receptor signal pathway in the pathogenesis of BL. Mutations of the transcription factor TCF3 or its negative regulator ID3 have been reported in about 70% of sBL cases. These mutations activate B-cell receptor signaling, which sustains BL cell survival by engaging the PI3K pathway. Other recurrent mutations, in CCND3, TP53, RHOA, SMARCA4, and ARID1A, occur in 5-40% of sBL/BAL. Both the number of mutations overall and the proportion of cases with mutations in TCF3 or ID3 are lower in endemic than sporadic BL. An inverse correlation between EBV infection and the number of mutations has been observed, sug-gestig that these mutations may serve in place of
the virus for the activation of the B-cell receptor signaling. It predict that these identified gene mutations play important roles in the pathopoiesis of BL/BAL. Genetic susceptibility individuals such as SH2D1A mutations are at greatly increased risk of developing BL; it is interesting that germline causal gene mutations were detected in four of the ten patients, but large sample and multiple centers are needed to verify the exact detection rate.

These mutated genes were enriched and connected by GO or KEGG pathway (Fig-2), it seemed that PI3K-Akt signaling pathway was the key role in the pathopoiesis of BL/BAL, similar finding was also analyzed by dataset and reported by literature review [16, 17]. BL/BAL may be inhibited by such signaling pathway [18].

Epidermal growth factor receptor (EGFR) is a tyrosine kinase; EGFR gene mutations and overexpression of its protein are associated with cancers growth [19]. Tyrosine kinase inhibitor (TKI) against EGFR (EGFR-TKI) has been administered for cancer patients with EGFR mutations, lung adenocarcinoma, i.e [20]. However EGFR-TKI resistance pathway was found by KEGG enrichment for identified genes in BL/BAL patients, it reveals that EGFR-TKI treatment is invalid in BL/BAL patients; further research is needed for the hypothesis and possible mechanisms.

**Conclusion**

BL/BAL is a highly aggressive but curable subtype of lymphoma, with combination treatment of intensive chemotherapy and Rituximab, the survival rate of BL/BAL has been improved steady; the pathophysiolopoiesis is still in research. The molecular hallmark of sBL/BAL is MYC translocation, whereas additional chromosomal abnormalities and gene mutations also occur and play roles in the progression of the disease.

In this study, NGS sequencing was detected and analyzed in pediatric sBL/BAL patients in Chinese population. Other recurrent mutations except MYC gene were detected, and possible signal pathways were also demonstrated, further lab studies will be carry on to prove it.

**Competing Interests**
The authors declare that there are no competing interests associated with the manuscript.

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**Author Contribution**

Jianwen Xiao and Jing Zhu conceived and designed the study. Ling Song and Nange Yin prepared the figures and tables. Xiaoying Lei analyzed and interpreted the data. Xiaoying Lei and Jianwen Xiao drafted the manuscript. Jianwen Xiao revised the manuscript. All authors read and approved the initial manuscript.

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