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Comparative genomic expression signatures of signal transduction pathways and targets in paediatric Burkitt lymphoma (PEBL): A Children's Oncology Group (COG) report

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Summary

Burkitt lymphoma (BL) is the most common histological subtype of non-Hodgkin lymphoma (NHL) in children and adolescents. Through the introduction of short intensive multi-agent chemoimmunotherapy, survival has improved significantly over the past 30 years. However, this successful approach is limited by significant chemotherapy-induced acute toxicity and risk of developing resistant disease, demonstrating the need to identify less toxic and targeted therapies. We analysed the comparative genomic signature and targetable signalling pathways in paediatric BL (PEBL) samples from the Children's Oncology Group study (ANHL01P1) by genomic profiling and selected genes were confirmed by quantitative real time polymerase chain reaction. These results were compared to PEBL samples from public databases and utilised the Gene Expression Omnibus (GEO) Series (GSE) 10172 and 4475 (n=16), and 4732 (n=15). Three hundred and seventy-six genes (approximately 25%) were similarly expressed among three PEBL sample groups. Several target genes in Toll-like receptor signalling, JAK-STAT signalling and MAPK signalling were significantly overexpressed in PEBL. In addition, several tyrosine kinases, including Bruton tyrosine kinase (BTK), protein tyrosine phosphatase (PTP) and histone

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deacetylase inhibitor (HDACi) were highly expressed in PEBL. These pre-clinical results suggest that specific signal transduction pathways are overly expressed in PEBL and several pathways could serve as potential future therapeutic targets.

Keywords

Gene expression profiles; Paediatric Burkitt lymphoma; Signal transduction pathways

Introduction

Paediatric Burkitt lymphoma (PEBL) is an aggressive mature B-cell malignancy; it is the most common (about 40%) non-Hodgkin lymphoma (NHL) in children and adolescents in western countries. The prognosis of PEBL has steadily improved over the past 30 years through the introduction of short and intensive multi-agent chemotherapeutic regimens (Cairo, *et al* 2007, Cairo, *et al* 2012, Gerrard, *et al* 2008, Miles, *et al* 2012, Patte, *et al* 2007). The Children's Oncology Group (COG), together with the United Kingdom Children's Cancer Group (UKCCSG) and the French Paediatric Oncology Group (SFOP), conducted a prospective international study in PEBL (French-American-British [FAB] 96) that demonstrated 90% 5-year overall survival (OS) including patients with stage III/IV disease and those with bone marrow (BM) and central nervous system (CNS) disease (Cairo, *et al* 2007, Cairo, *et al* 2012, Gerrard, *et al* 2008, Patte, *et al* 2007). More recently, we have demonstrated the safety and efficacy of adding rituximab to the chemotherapy backbone (FAB 96) in children and adolescents with advanced mature Burkitt lymphoma (BL) (COG ANHL01P1) (Goldman, *et al* 2013, Goldman, *et al* 2014). However, this success has come at the cost of the significant chemotherapy-induced acute toxicity secondary to intensive chemotherapy, requiring the need to identify less toxic but targeted therapy. Furthermore, patients with PEBL who relapse or progress have chemotherapy-resistant disease and can rarely be salvaged (Cairo, *et al* 2007, Cairo, *et al* 2012, Miles, *et al* 2012). These results suggest the urgent need to identify alternative treatment approaches focusing on targeted therapy to circumvent chemotherapy-resistant disease by providing alternative therapeutic targets.

The rearrangement of proto-oncogene *MYC* on 8q24 is the hallmark cytogenetic feature of BL (Dave, *et al* 2006, Nelson, *et al* 2010, Poirel, *et al* 2009). The activation of *MYC* has been implicated in the promotion of aberrant cell proliferation (Shaffer, *et al* 2002). A set of *MYC* target genes were identified in both paediatric and adult BL samples by comparing all subtypes of diffuse large B-cell lymphoma (DLBCL) with samples of BL (Dave, *et al* 2006, Deffenbacher, *et al* 2012, Giulino-Roth, *et al* 2012, Hummel, *et al* 2006, Klapper, *et al* 2008). These results suggested that BL, in part, uses different oncogenic signalling pathway(s) compared to other B-cell lymphomas, including DLBCL. Unlike many other haematological malignancies, the nuclear factor (NF)-kB pathway was not constitutively activated in BL (Dave, *et al* 2006) and recently, the phosphatidylinositol 3-kinase (PI3K) pathway has been suggested as a key signalling pathway in BL lymphomagenesis (Sander, *et al* 2012). However, it is still unclear whether diverse signalling pathway(s) contribute to paediatric Burkitt lymphomagenesis. While it is important to know the degree of variability

in the pathogenetic networks, a fuller understanding of these, as well as the key points in the pathways, could lead to more rational target selection than simply identifying the pathway of genes in the molecular signature. In this study we analysed the comparative genomic signatures of PEBL among COG PEBL samples from the most recent COG PEBL Study (COG ANHL01P1) and compared our results with the databases of Berlin-Frankfurt-Muenster (BFM) NHL samples (Klapper, *et al* 2008) and National Cancer Institute (NCI) samples (Dave, *et al* 2006). This approach demonstrated several potential targetable signalling pathways in PEBL.

Materials and methods

Patient samples

Primary tumour specimens were obtained from children and adolescents with newly diagnosed stage III/IV or BM ± CNS BL registered on the COG ANHL01P1 study (Clinicaltrials.gov NCT00057811) (Goldman, *et al* 2013, Goldman, *et al* 2014) that were submitted to the reference laboratory at Columbia University. The protocol was approved by the institutional review board of each COG centre participating in this trial. Samples (1–2 ml) of PEBL patient-involved BM, pleural fluid, peritoneal fluid and/or lymph node with tumour involvement were collected at the time of diagnosis. The specimens for molecular studies were immediately submerged in RNAlater reagent (Qiagen, Valencia, CA), and shipped overnight at room temperature to the central research laboratory at Columbia University. Upon receipt, the specimens in RNAlater were immediately processed to extract total RNA and frozen at −80°C.

Haematopathology

All cases underwent central pathology review for confirmation of diagnosis, consisting of review of morphology, immunophenotype and genetic data from the original diagnostic biopsy. If required, additional immunophenotyping was performed centrally to confirm the diagnosis according to the 2008 World Health Organization Classification (Swerdlow, *et al* 2008). In addition, CD20 expression of the tumour was confirmed by immunohistochemistry (L-26 clone, DAKO Cytomation, Carpinteria, CA) using standard methods and heat-induced epitope retrieval in citrate buffer (pH6.0) on an automated stainer (ES, Ventana Medical Systems, Tucson, AZ). A tumour was scored as CD20 positive if >80% of the tumour cells stained with the antibody.

Cytogenetics and fluorescence in situ hybridization (FISH) analysis

Cytogenetic analysis was performed, as previously described, with nomenclature according to the International System for Human Chromosome Nomenclature (Poirel, *et al* 2009, Shaffer and Tommerup 2005). Interphase FISH analysis for *MYC* rearrangement was performed on a portion of the specimen submitted for cytogenetic analysis. A dual-colour *MYC/IGH* translocation probe designed to detect t(8;14)(q24.1;q32) or a dual-colour *MYC* break-apart probe, designed to detect rearrangements of the *MYC* gene region at 8q24.1 with various partner chromosomes, was utilised according to the COG reference laboratories standard protocols. Slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in

Antifade solution and the nuclei were visualized and captured using a fluorescence microscope equipped with appropriate filters and imaging software.

Clinical characteristics

Specimens were collected from 11 PEBL patients registered on the COG ANHL01P1 study and the clinical and demographic data on these patients are described in Table I. All patients were treated with chemoimmunotherapy with modified FAB B4 and C1 chemotherapy with the addition of rituximab in induction and consolidation as previously described (Barth, *et al* 2013, Galardy, *et al* 2013, Goldman, *et al* 2013, Goldman, *et al* 2014, Shiramizu, *et al* 2011).

Oligonucleotide cDNA microarray analysis

RNA purification and hybridization for the microarray analyses were performed as previously described (Jiang, *et al* 2009). In brief, total RNA from patient tumour specimens was isolated by Trizol reagents (Invitrogen, Carlsbad, CA) and followed by affinity column purification (Qiagen, Valencia, CA). cDNA was generated from 100 ng total RNA using a poly dT oligonucleotide (Affymetrix, Santa Clara, CA). Biotinylated labelling of cRNA was performed by *in vitro* transcription according to manufacturer's protocol. Fragmented cRNA (15 μg) was hybridized to HG_U133 A2 Gene Chip® arrays (Affymetrix) using Fluidics Station 450 (Affymetrix).

Gene expression profiling

Signal intensities of gene expression were calculated by Expression Console (Affymetrix). Data were imported into GeneSpring GX 10 (Agilent Technologies, Foster City, CA) or Partek Genomics Suite (St. Louis, MI) and normalised and presented as $\log^2$ values. Fold changes of target expression were compared to the average of 530 median expressed genes. The identified gene information and accession numbers were first obtained through NetAffx, provided by Affymetrix, and then each entity was further confirmed by a search of GenBank. Data has been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/; accession number GSE64905).

Quantitative real time polymerase chain reaction (qRT-PCR)

Selected genes (*PIM1, STAT1, MAP2K1, RAF1, PTPN11, DLEU1*, and *MYC*), based on being a BL classifier gene or representing different signalling transduction pathways, were examined for their expression levels by qRT-PCR using qScript™ cDNA Synthesis Kit (Quantas, Gaithersburg, MD) and SsoFast™ EvaGreen® Supermix (Bio-rad, Hercules, CA). The primer sequences are listed in Supplementary Table I. Real time detection of the RT-PCR product was monitored using the CFX96 Real-time system (Bio-rad). Relative quantification (ddCt) of mRNA expression of each gene was determined by normalizing to the housekeeping gene *GAPDH*. cDNA amplification efficiencies of target and reference genes were confirmed to be equivalent and the absolute values of the slope of log input amount vs. ΔC_T were < 0.1. Fold changes were obtained by being normalised to the qRT-PCR results of *GAPDH* expression.

Comparative genomic signature

For the genomic comparison in this study, paediatric genome expression results from three different research groups were utilised (Supplementary Table II); COG ANHL01P1 in the current study (n=11), GEO GSE10172 and GSE4475 (Klapper, *et al* 2008) (n=16), and GSE4732 (Dave, *et al* 2006) (n=15) (≤13 years of age).

Pathway and function analysis

Functional interpretation of the classified genes with similar expression from the comparative genomic data of COG, NCI and BFM, and relevance to molecular and cellular functions and pathways was generated by Ingenuity Pathways Analysis (IPA®), version 6.5, (Qiagen, Redwood City, CA). P-values less than 0.05 indicate a statistically significant and non-random association between a set of computational selected genes from the 376 PBL genes and a set of all genes related to a given function in Ingenuity's knowledge base.

Gene functional classification

The genes using Affymetrix ID as the identifier were uploaded into the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) at the National Institute of Health, and then analysed by functional annotation clustering. Gene lists obtained from each functional group were then re-uploaded into DAVID, and then searched for specific pathways.

Statistical analysis

Statistical differences between the three genomic expression profiles were analysed by Student *t* test; differences between multiple groups were analysed by one-way ANOVA followed by the Tukey multiple comparison test. Results are expressed as mean±standard error of the mean (SEM) with probability values less than or equal to 0.05 considered significant.

Results

Demographics

The mean age of the COG BL patients was 11.3 years, with a male/female ratio of 9:2. There were 3 stage III and 8 stage IV patients. Seven patients had BM involvement (≥25% L3 blasts) and one patient had CNS disease. Four patients were treated on the group B arm and seven patients were treated on the group C arm (Table I) (Goldman, *et al* 2013, Goldman, *et al* 2014).

Haematopathology

All of the cases used in this study showed typical morphological and immunophenotypic features of BL, including CD20, CD10 and BCL6 positivity and lack of expression of T-cell markers and BCL2 protein. In all cases, the tumours showed a high proliferative rate (>95%) by Ki-67 immunohistochemistry.

Cytogenetics

Six of 11 patients had evaluable cytogenetics. One patient was FISH positive for *IGH/MYC* but had a failed karyotype. Three patients had a complex karyotype, defined as the presence of 3 or more chromosomal aberrations, and three patients had a t(8;14)(q24;q32) translocation (Supplementary Table III).

Comparative genomic signature

A total of 1565 genes were identified ($p<0.05$), among which 376 genes showed a similar genomic expression pattern within the PEBL signature among the three clinical subgroups (Fig. 1). *MYC*, a molecular identifier for BL, showed 27-fold greater expression in these subgroups, consistent with the previous BL signature described by Dave et al. (2006). We further classified these 376 genes and identified 12 major functions (333 genes with known functions and 43 genes with unknown functions) that were involved in PEBL (Figs. 2A and 2B). Detailed classifications and descriptions of genes in each 12 functional group are listed in Supplementary Table IV. The lead functional categories were serine/threonine kinases and tyrosine kinases (87 genes), cytokine binding receptors (80 genes) and transcription (77 genes) (Figs. 2A and 2B). PEBL tumour samples also demonstrated significantly higher levels of expression of genes, including the protein tyrosine phosphatases (PTP), *PTPN11* (also called *SHP2*; 36.6-fold), and *PTPN6* (8.6-fold) *SHP1*). Small GTPases and regulators were also significantly expressed in PEBL (10 genes), including *RALBP1* (8.9-fold), *RHOG* (9.7-fold), *ARHGAP19* (9.7-fold), *ARHGEF18* (21.5-fold) and *ARHGAP17* (8.9-fold). Seven glycolysis genes were also significantly overexpressed (8.1- to 109.5-fold).

Comparison of genomic profiling and qRT-PCR

To validate the level of gene expression generated from oligonucleotide microarray studies, we analysed a selected number of target genes with significant clinical implication and/or biological function by qRT-PCR. The qRT-PCR results were then compared to the results of microarray analysis. These genes were *PIM1, STAT1, MAP2K1, RAF1, PTPN11, DLEU1* and *MYC*, and the fold changes by qRT-PCR were 4.7, 10.3, 8.7, 21.3, 35.0, 8.1 and 17.2, respectively (Fig. 3). These qRT-PCR results were consistent with gene expression studies generated from the microarray studies.

Targetable signalling pathways

Within the 376 genes identified as common in all three data sets, IPA® demonstrated a significant association with three signalling pathways - Toll-like receptor (TOLL) signalling ($p<0.01$), Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling ($p<0.01$), and mitogen-activated protein kinase (MAPK) signalling ($p<0.01$) pathways were significantly overexpressed (Fig. 4 and Supplementary Table V). We identified gene overexpression of 7 kinases involved in the TOLL pathway and 13 kinases involved in the MAPK pathway from 87 serine/threonine kinases and tyrosine kinases (Supplementary Table VI), and 6 receptor genes involved in the TOLL pathway, JAK-STAT pathway and cytokine binding receptors pathway. Expression of genes involved in the TOLL signalling (Supplementary Table VII) included *TLR7* (2.9-fold), *IRAK1* (34.1-fold), *NFKBIA* (18.1-fold) and *IKBKB* (7.2-fold). In addition, the genes encoding interleukin

(IL)-10 receptor (*IL10RA;* 3.1-fold) and IL-21 receptor (*IL21R*; 2.3-fold) were both expressed at a similar level to *TLR7*. Expression of genes involved in JAK-STAT signalling (Supplementary Table VII) included *PIM1* (8.7-fold), *STAT1* (11.1-fold), *TYK2* (4.1-fold) and *IFNAR2* (6.2-fold) and expression of genes involved in the MAPK signalling in PEBL included *MAP2K1* (11.1-fold), *MAP3K7* (7.6-fold), *MAPK9* (10.5-fold), *MAP4K4* (8.3-fold), *RAF1* (11.3-fold), *PTPN11* (36.6-fold) and *PTPN6* (8.6-fold). Most interestingly, the B cell receptor signalling (BCR) pathway had a significant overexpression of *BTK* (9.1-fold) and a significant increase in *HDAC1* (42-fold) and *HDAC2* (15.5-fold) (Supplementary Table V).

Discussion

This is the first report using comparative genomic identification to compare the genomic expression signature in PEBL. We compared our COG PEBL samples with PEBL samples reported by the NCI (Dave, *et al* 2006) and the BFM (Klapper, *et al* 2008). These data suggested that PEBL gene expression signatures are consistent among three cooperative groups/laboratories. The fold changes in PEBL gene expression profiles in these three sets of samples were highly similar, if not identical. In addition, the comparative data was remarkable given that these three groups used different designated genechips for microarray. The samples investigated by Dave, *et al* (2006) were processed by custom-made cDNA Lymphochips, while Klapper *et al* (2008) and our group both utilised Affymetrix Oligonucleotide Genechips (Jiang, *et al* 2009).

Our results indicated that TOLL, MAPK, and JAK-STAT signalling pathways are overexpressed in PEBL samples, including threonine kinases, tyrosine kinases and cytokine binding receptors that regulate cellular proliferation (Supplementary Table VI). Interestingly, seven genes involving the regulation of glycolysis (Vander Heiden, *et al* 2009) were significantly overexpressed in PEBL (Fig. 3), and several target genes involved in the TLR signalling pathway (Supplementary Table VI), *TLR7* (2.9-fold), *IRAK1* (34.1-fold), *NFKBIA* (18.1-fold) and *IKBKB* (7.2-fold), were significantly overexpressed. In addition, *IL10RA* (3.1-fold) and IL21R (2.3-fold) were both expressed at a similar level to *TLR7*. The TLRs and the IL1 receptor (IL1R) are known to possess a highly conserved intracellular Toll-IL-1R (TIR) domain (Barton and Medzhitov 2003). Furthermore, TLR triggering has been shown to regulate cellular growth in BL (Noack, *et al* 2012) and TLR activation has been implicated in tonic BCR activation in DLBCL (Akhter, *et al* 2015). Ligand activated TLRs cause dimerization of the cytoplasmic TIR domains, which significantly activate the IL-1R associated kinase 1 (i.e. IRAK1) and then further activates the downstream NF-κB signalling. The consequence of this TLR pathway is to enhance the production of interferons (IFNα and IFNβ) in PEBL. MAPK has been demonstrated to be overexpressed in EBV-positive vs EBV-negative BL (Cerimele, *et al* 2005).

Target genes involved in JAK-STAT signaling (Supplementary Table VI) included *PIM1* (8.7-fold), *STAT1* (11.1-fold), *TYK2* (4.1-fold) and *IFNAR2* (6.2-fold), which were also significantly overexpressed. The oncogenic *PIM1* has been implicated as a critically important gene with *MYC* in the pathogenesis of a number of haematological malignancies (Wang, *et al* 2010). Furthermore, these preclinical data suggest that the interferons may

activate *IFNAR2* in PEBL and, in turn, IFNAR2 activates *TYK2*. The activated *TYK2* then phosphorylates STAT1 and STAT4 and may result in dimerization of these transcription factors. The STAT1 and STAT4 dimers may then translocate into the nucleus where they bind on the promoter region of *PIM1*. This pathway may be partly responsible for PEBL lymphomagenesis.

Many MAP kinase signalling pathway genes were significantly overexpressed; target genes involved in MAPK signalling in PEBL (Supplementary Table VII) included *MAP2K1* (11.1-fold), *MAP3K7* (7.6-fold), *MAPK9* (10.5-fold), *MAP4K4* (8.3-fold), *RAF1* (11.3-fold), *PTPN11* (36.6-fold) and *PTPN6* (8.6-fold). MAPKs are serine-threonine kinases that regulate a wide variety of cellular functions (Johnson and Lapadat 2002). *RAF1* encodes a MAP kinase kinase kinase (MAP3K). Mitogen-activated protein kinase (MAPK9), also known as a c-Jun N-terminal kinase (JNK2), blocks the ubiquitination of tumour suppressor TP53 (Johnson and Lapadat 2002). PTPN11 and PTPN6 are protein tyrosine phosphatases (Alonso, *et al* 2004, Ostman, *et al* 2006). From these studies we hypothesise that PEBL cells express IFNα or IFNβ following induction of TOLL signalling pathways and the overexpression of *IRAK1* results in an increase of the TOLL signalling pathway. In addition, an increase of *MAP3K7* may enhance the phosphorylation of IKK. These augment phosphorylation of IκBα and IκBβ and release of NFκB, then dimerized NFκB bind to the IFNα and IFNβ promoters in the nucleus and promote IFNα and IFNβ production. These interferons then activate the JAK-STAT and MAPK signalling pathways. Although the functions of these interferons are to enhance apoptosis and reduce proliferation, a highly activated RAS-MAPK signalling pathway enhances oncogene activation, including *MYC* (Roux and Blenis 2004). The interferon-activated IFNAR2-mediated MAPK signalling pathway in PEBL, via RAS proteins, phosphorylates *MYC* and enhances cellular proliferation and promote anti-apoptosis. Furthermore, serine/threonine kinases and tyrosine kinases were significantly overexpressed in this PEBL genomic study. From these comparative genomic signatures, we have demonstrated that *IRAK1, RAF1, MAP4K4, BTK, MAP3K7, MAP2K1, MAPK9, NFKBIA, IKBKB, PIM1, STAT1, PTPN6*, and *PTPN11* may be candidate targets in the treatment of PEBL. Currently, there are several clinical trials using inhibitors/siRNA of targets in BL in phase I studies, including *BTK* by ibrutinib (NCT0119069), *RAF1* inhibition by regorafenib (NCT02693535) and *MYC* inhibition by siRNA DCR-MYC (NCT02110563). Additionally, we recently demonstrated that proteins with cellular functions including NF-kB and MAPK signalling pathways are significantly overexpressed in different epidemiological subtypes of BL cell lines compared to normal B cells from healthy donors by proteomic expression profile studies (ElMallawany, *et al* 2014).

More interestingly, we have observed a significant increase of BCR signalling pathway genes, including *BTK*, and have recently demonstrated that the inhibition of PI3K/Akt is a key in a Burkitt lymphomagenesis (Frys, *et al* 2014) (Ippolito, *et al* 2015). BTK is a regulator of normal B-cell development and is activated upon BCR stimulation. Mutations of *TCF3* and it's negative regulator *ID3*, including *MYC*, are prevalent in BL and contribute to activation of the BCR pathway (Schmitz, *et al* 2012) (Love, *et al* 2012) and interestingly, *MYC* transcriptional expression suppressed small molecule inhibitors of the BET family of chromatin adaptors (Mertz, *et al* 2011). However, BL tumourigenesis may be secondary to

dysregulation of un-mutated genes via transcription factors versus mutations in the targets of transcription factors or alteration of the function of other genes on the signalling pathway.

Chronic active BCR signalling through BTK activation can be inhibited by the selective, covalent BTK inhibitor, ibrutinib (Davis, *et al* 2010, Young and Staudt 2013). Ibrutinib is an active agent in activated B cell like DLBCL (Mathews Griner, *et al* 2014), and preclinical studies in chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma (MCL) suggested an inhibitory effect on cell proliferation (Cinar, *et al* 2013, Dasmahapatra, *et al* 2013, Honigberg, *et al* 2010, Ponader, *et al* 2012). Furthermore, we have recently demonstrated that ibrutinib has a significant inhibitory effect of on cell proliferation and increase in caspase 3/7 and apoptosis in BL (Lee, *et al* 2015). Clinically, ibrutinib has been highly effective in the treatment of refractory patients with CLL and MCL and has been approved by the Food and Drug Administration for patients with CLL or MCL who have received at least one prior therapy (Byrd, *et al* 2013, Wang, *et al* 2010, Wang, *et al* 2013).

In summary, this is the first study comparing the genomic signature of PEBL from three completely independent databases. Among the 1565 genes identified, 376 genes (approximately 25%) were similarly expressed between the 3 groups. Furthermore, there was significant overexpression of genes involved in the Toll receptor, JAK-STAT and MAPK signalling pathways. Among these pathways, several genes, including *IRAK1, PTPN6, BTK, RAF1, HDAC1 and HDAC2* may be attractive targets for alternate targeted therapeutic strategies in PEBL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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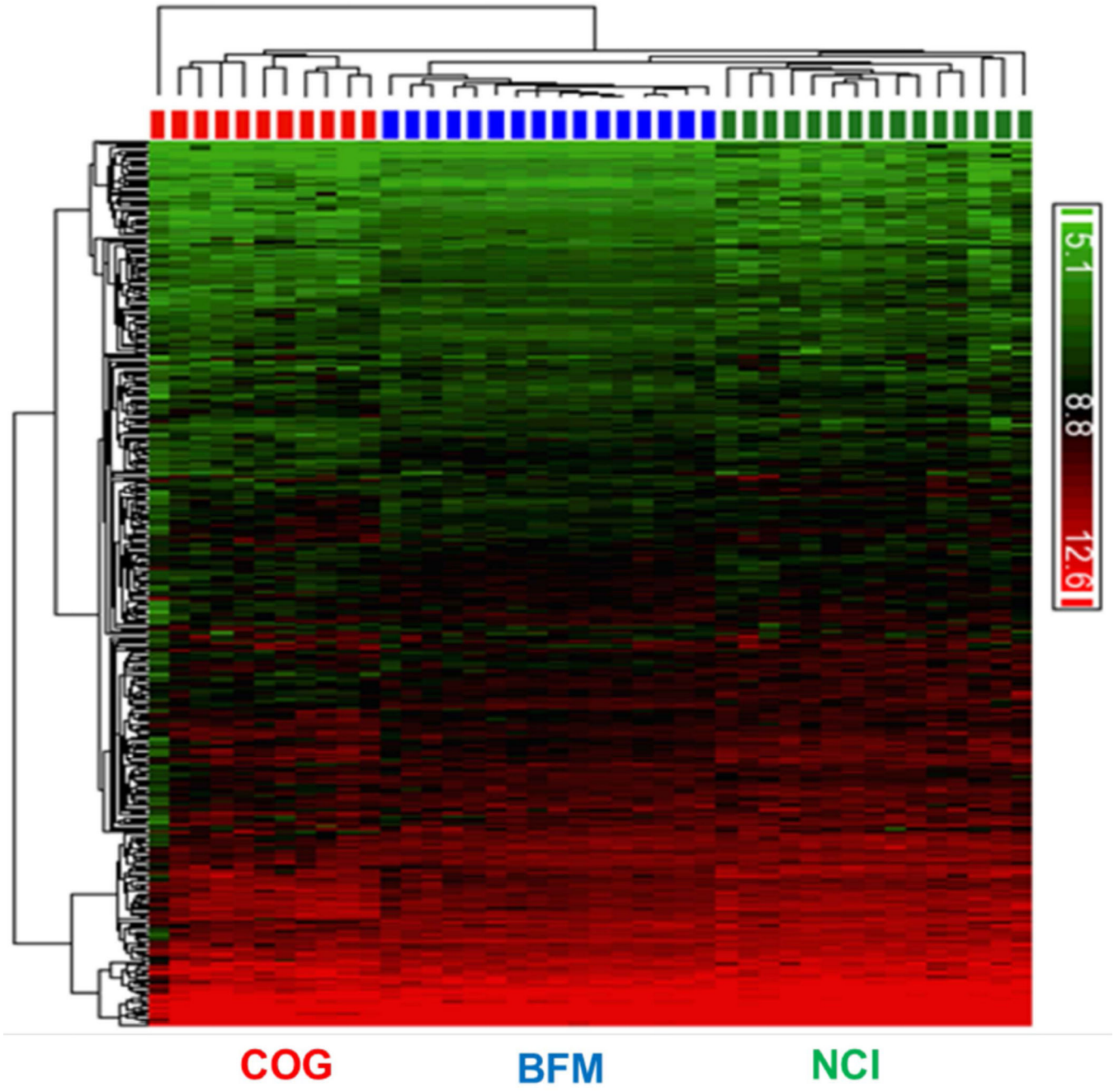


Figure 1. Hierarchical clustering displaying gene expression patterns in paediatric Burkitt lymphoma

Similarly expressed genes were compared between three paediatric Burkitt lymphoma clinical subgroups (COG, Children's Oncology Group (ANHL01P1 study); BFM (Klapper, *et al* 2008), NCI (Dave, *et al* 2006) demonstrating green (decreased expression) and red colour (increased expression) according to the scale shown. Detailed procedures are described in the Methods.

COG, Children's Oncology Group; BFM, Berlin-Franfürt-Münster; NCI, National Cancer Institute

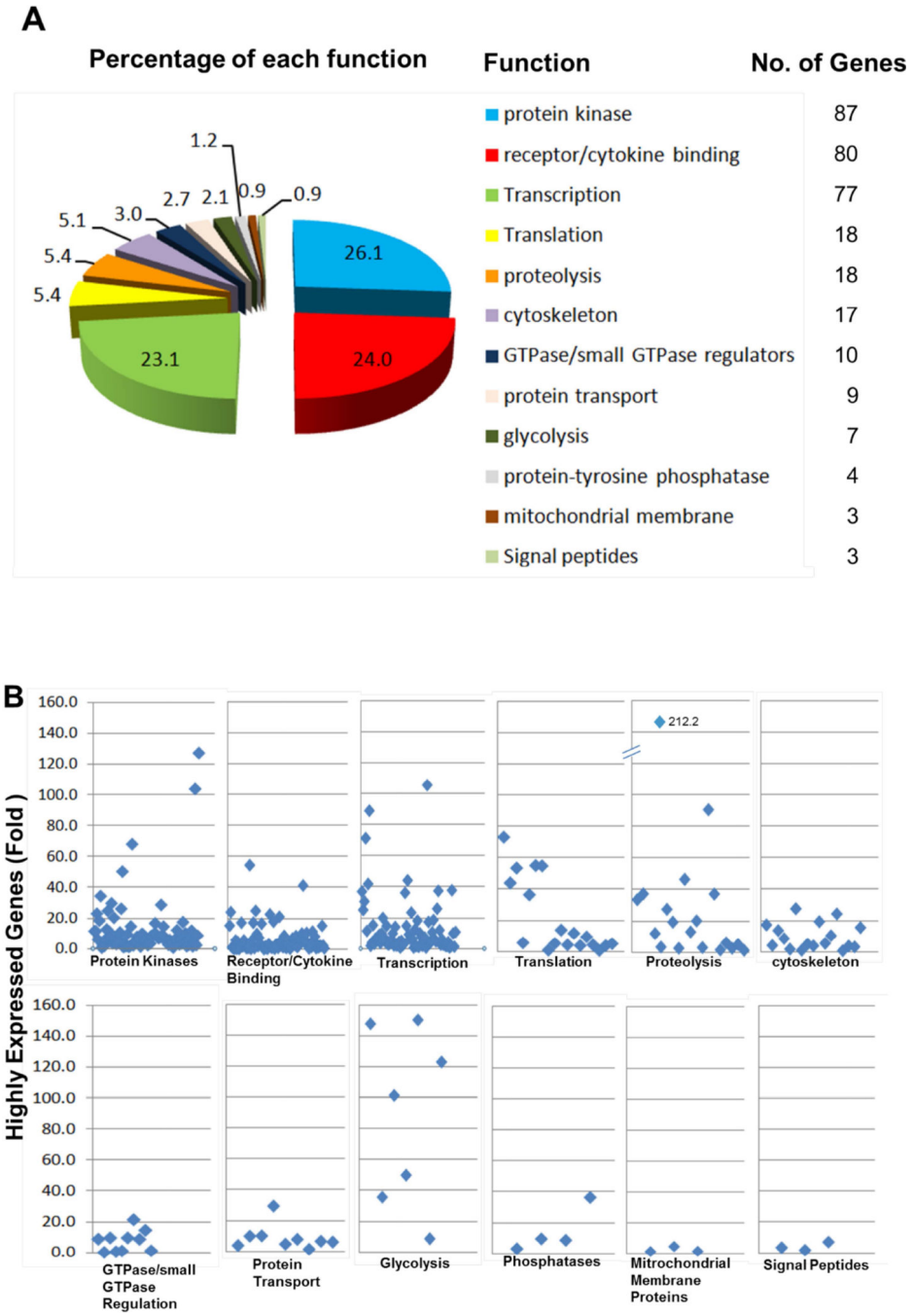
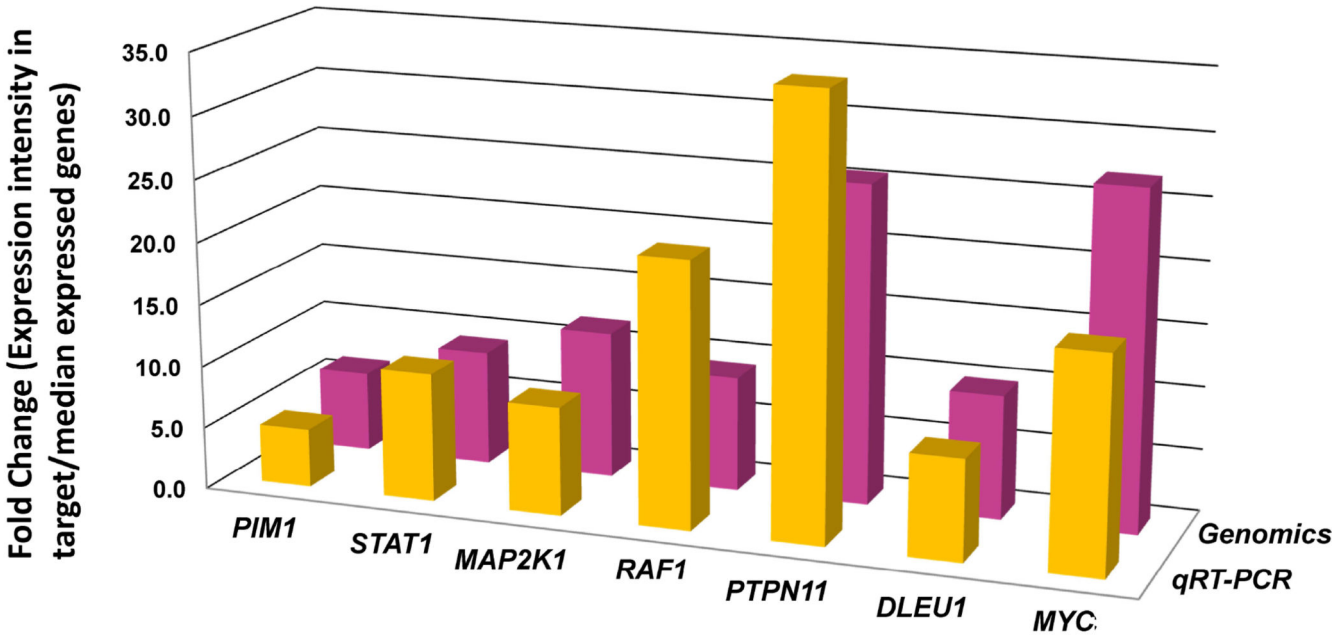


Figure 2. Expression of genes in paediatric Burkitt lymphoma samples stratified by functional group

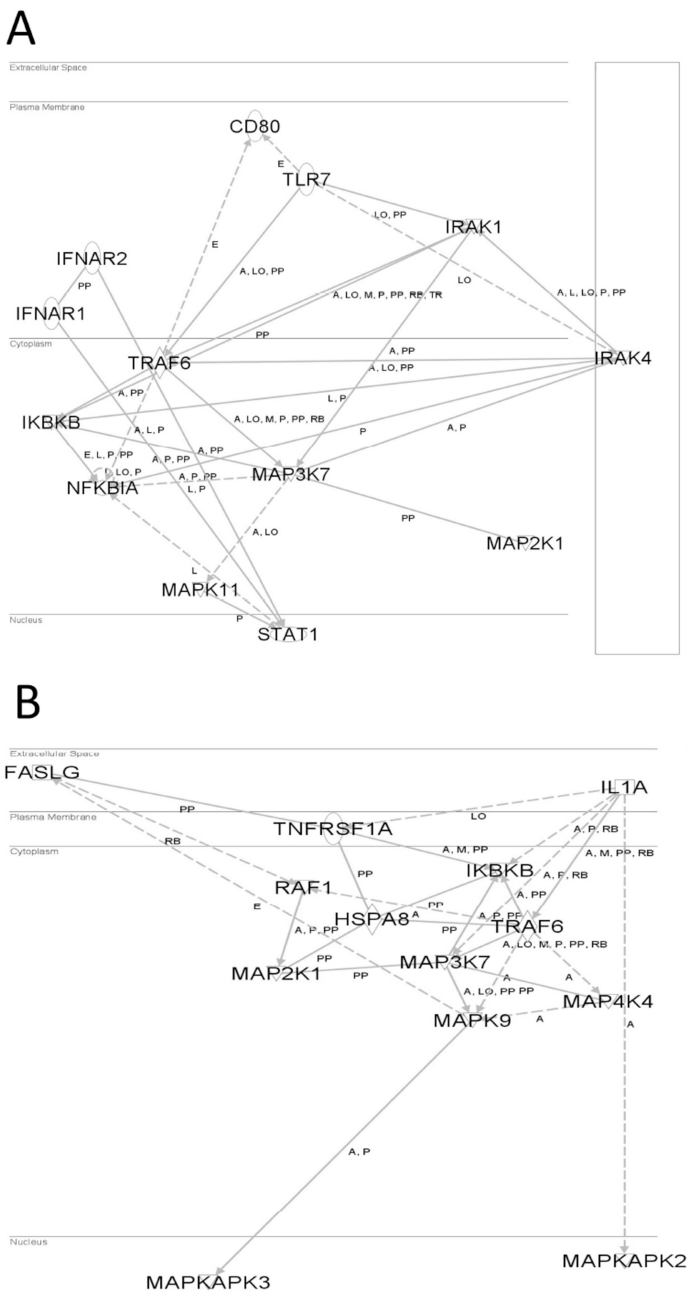
A total of 376 similarly expressed genes were grouped into 12 functional categories. The percentage (A) and fold changes and numbers (B) of genes in each functional category are demonstrated.



		PIM1	STAT1	MAP2K1	RAF1	PTPN11	DLEU1	MYC
Fold change	qRT-PCR	4.7	10.3	8.7	21.3	35.0	8.1	17.2
Fold change	Genomics	6.4	9.2	11.8	9.2	25.6	9.9	27.0

Figure 3. Comparison of fold changes in gene expression in paediatric Burkitt lymphoma samples between Affymetrix Genechip profiling and real time qRT-PCR analyses of *PIM1, STAT1, MAP2K1, RAF1, PTPN11, DLEU1* and *MYC* genes

Quantitative real time polymerase chain reaction (qRT-PCR) expression levels of each selected genes are consistent with the results of genechip expression profiling analysis on Paediatric Burkitt Lymphoma samples. The fold changes were calculated from 27 and 9 PEBL samples for genomics and qRT-PCR, respectively, and histogram show the data of qRT-PCR with the means of three independent experiments performed in duplicate. *GAPDH* was used as endogenous control for qRT-PCR normalization.



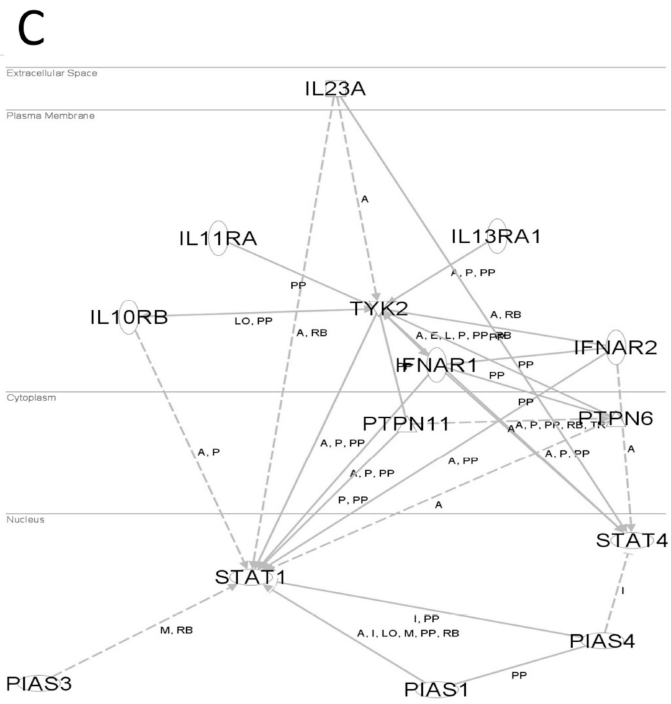


Figure 4. Ingenuity pathway analysis of gene networks in TOLL-like receptor (A), MAPK (B), and JAK-STAT3 (C) pathways on paediatric Burkitt lymphoma samples

Three significant signalling pathways were identified by the ingenuity pathway analysis tool (www.ingenuity.com) using up-regulated genes in paediatric Burkitt lymphoma samples in Toll-like receptors (TLRs), Mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways.

A, activation; E, expression; I, inhibition; L, proteolysis; LO, localisation; M, biochemical modification; P, phosphorylation/dephosphorylation; PP, Protein-protein binding; RB, regulation of binding; TR, translocation.

Table I

Clinical Characteristics of COG Patients

Patient	Age (Years)	Gender	Histology	Stage	FAB Group	Bone Marrow (≥25% blasts)	CNS Status
001	7.5	Male	Burkitt	Stage IV	Group C	Positive	Positive
002	1.9	Female	Burkitt	Stage III	Group B	Negative	Negative
003	14.5	Female	Burkitt	Stage IV	Group C	Positive	Negative
004	9.5	Male	Burkitt	Stage III	Group B	Negative	Negative
005	15.0	Male	Burkitt	Stage III	Group B	Negative	Negative
006	11.9	Male	Burkitt	Stage IV	Group C	Positive	Negative
007	8.1	Male	Burkitt	Stage IV	Group C	Positive	Negative
008	10.5	Male	Burkitt	Stage III	Group B	Negative	Negative
009	18.8	Male	Burkitt	Stage IV	Group C	Positive	Negative
010	11.8	Male	Burkitt	Stage IV	Group C	Positive	Negative
011	15.0	Male	Burkitt	Stage IV	Group C	Positive	Negative

COG, Children's Oncology Group; FAB, French-American-British classification; CNS, central nervous system.