Malaysian J Pathol 2019; 41(3) : 273 – 281

ORIGINAL ARTICLE

pSTAT3 and MYC in Epstein-Barr virus-positive diffuse large B-cell lymphoma

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Abstract

Introduction: Epstein-Barr Virus (EBV) is associated with several B-cell non-Hodgkin's lymphoma (NHL), but the role of EBV in diffuse large B-cell lymphoma (DLBCL) is poorly defined. Several studies indicated the expression of phosphorylated STAT3 (pSTAT3) is predominant in EBV(+)-DLBCL, of which its activated form can promote the downstream oncogenes expression such as *c-MYC*. *c-MYC* gene rearrangements are frequently found in aggressive lymphoma with inferior prognosis. Furthermore, EBV is a co-factor of MYC dysregulation. JAK1/STAT3 could be the downstream pathway of EBV and deregulates MYC. To confirm the involvement of EBV in JAK1/STAT3 activation and MYC deregulation, association of EBV, pSTAT3 and MYC in EBV(+)-DLBCL cases were studied. The presence of pSTAT3 and its upstream proteins: pJAK1 is identify to delineate the role of EBV in JAK1/STAT3 pathway. *Materials and Methods:* 51 cases of DLBCL paraffin-embedded tissue samples were retrieved from a single private hospital in Kuala Lumpur, Malaysia. EBER-ISH was performed to identify the EBV expression; ten EBV(+)-DLBCL cases subjected to immunohistochemistry for LMP1, pJAK1, pSTAT3 and MYC; FISH assay for *c-MYC* gene rearrangement. *Results:* Among 10 cases of EBV(+)-DLBCL, 90% were non-GCB subtype (p=0.011), 88.9% expressed LMP1. 40% EBV(+)-DLBCL had pJAK1 expression. *Conclusion:* 66.7% EBV(+)-DLBCL showed the positivity of pSTAT3, which implies the involvement of EBV in constitutive JAK/STAT pathway. 44.5% EBV(+)-DLBCL have co-expression of pSTAT3 and MYC, but all EBV(+)-DLBCL was absence with *c-MYC* gene rearrangement. The finding of clinical samples might shed lights to the lymphomagenesis of EBV associated with non-GCB subtypes, and the potential therapy for pSTAT3-mediated pathway.

Keywords: EBV, DLBCL, EBV(+)-DLBCL, pJAK1, pSTAT3, MYC

INTRODUCTION

Epstein-Barr virus-positive diffuse large B-cell lymphoma (EBV(+)-DLBCL) is one of the EBV-associated lymphoproliferative disease, defined as EBV(+)-DLBCL, NOS in 2016 WHO classification. Majority of EBV(+)-DLBCL patients are elderly due to their senescence of immune system, but young adults cases also been reported. The incidence of EBV(+)-DLBCL is various ranging from 5% to 15%. For Asian countries, it was reported to have higher frequency (9-15%), whereas less than 5% in western countries.[1-2] EBV(+)-DLBCL also possess poor prognosis when compared to EBV(-)-DLBCL patients, but in western countries, the outcome is controversial.[3]

EBV(+)-DLBCL is categorised in latency type III when the board spectrum viral genome is expressed. Latency type III also known as a growth program, where EBV infected primary B cells and drives them to the proliferation cycle. One of the EBV latent genome latent membrane protein-1 (LMP1), is an oncoprotein where activates multiple signaling pathways. Several studies indicate LMP1 up-regulates cytokines interleukin 6 (IL6), and subsequently induce the

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JAK/STAT3 signaling pathway. Activation of STAT3 translocates to the nucleus and promotes the regulation of *c-MYC* gene. Furthermore, EBV viral genome can render the activation of the *c-MYC* gene rearrangements. In the study of *c-MYC* gene rearrangement in DLBCL, poor prognosis of regimen R-CHOP treatment was discovered for the patients in conjunction with *BCL2* and/or *BCL6* translocation (Double-hit and Triple-hit lymphoma).[4] *c-MYC* gene rearrangement also found in 7-15% of *de novo* DLBCL, and 8% of post-transformation DLBCL, this has supported that *c-MYC* genes have highly occurred in high-grade lymphoma. Moreover, higher incidence of *c-MYC* gene rearrangement and Double-hit lymphoma were observed in EBV(+) cases in Asian countries.[5-7]

STAT3 is an upstream receptor of MYC. It also known as a transcription factor in response to cytokines and growth factors. Studies found that phosphorylated STAT3 (pSTAT3) is predominantly actives in EBV(+)-DLBCL. pSTAT3 form homo- or heterodimers and translocate to nucleus, involve in the cell survival and proliferation. Constitutively active STAT3-mediated pathway can regulate downstream oncoproteins, such as MYC.[8-9] NF-κB and STAT3 are activated in EBV related tumours.[10] Therefore, it was hypothesised in this study that the aberration of STAT3 was caused by the viral latent protein LMP1 and resultsed in MYC upregulation.

MYC is frequently found in aggressive lymphoma phenotype. In B-cell aggressive NHL, MYC is the hallmark of BL. MYC expression is commonly associated with EBV infection in BL, but studies on the association between EBV and MYC in aggressive DLBCL have been limited. For MYC expression in DLBCL, newly defined high grade B-cell lymphoma in 2016 WHO classification with concurrent genes alteration of *c-MYC, BCL2* and *BCL6* were also reported to have worse outcome, especially for the patients with *c-MYC* translocation.[11]

An association study between EBV, JAK1/STAT3 and *c-MYC* gene and protein may suggest the involvement of the JAK1/STAT3 pathway in EBV(+)-DLBCL has caused the MYC deregulation. Immunohistochemistry staining is by far still the more economic and rapid diagnostic tools in clinical labs, and our finding can throw light on the utility of this test in targeting pSTAT3 in therapeutic agent, as well as the studies of EBV in B-cell pathogenesis.

MATERIALS AND METHODS

An archival of 51 DLBCL cases diagnosed between 2011 and 2015 were retrieved from Pantai Premier Pathology in Kuala Lumpur, Malaysia with UCSI University Faculty of Medicine and Health Science Ethics Committee approval and complied with the Helsinki Declaration 2008. 3μm sectioned samples by Leica microtome from formalin-fixed paraffin-embedded (FFPE) tissue block, was subjected to EBER-ISH, IHC and 2μm of sectioned for FISH assay.

EBER *In Situ* Hybridization (EBER-ISH)

FFPE tissue were sectioned at 3μm, baked and mounted on Leica BOND Plus Slide (Leica Biosystems Richmon, Illinois, USA). EBV RNA in nucleus was detected by fluorescein-conjugated oligonucleotide probe supplied in hybridisation solution (BOND™ Ready-to-Use ISH, Leica). Slides were hybridised with the probe for 5 hours and amplified with BOND™ Polymer Refine Detection kit. The procedure was carried out as suggested by the manufacturer of the automatic Leica BOND MAX system immunostainer. RNA-positive and RNA-negative control probe were used to ensure the quality of mRNA. RNA-positive control probe is an oligonucleotide aim to hybridise Poly-A tail of mRNA for detection of preserved mRNA. All results were examined under 100X magnification Olympus microscope for scoring by experienced pathologist. Positive scoring was given if ≥50% of EBER expression detected in tumour cells.

Immunohistochemistry (IHC)

Tissue slides were de-paraffinised and rehydrated accordingly. Prior to the staining procedure, the slides were applied on Leica BOND MAX system immunostainer according to the manufacturer's procedure. A series of primary antibodies: CD10 Ab2 (56C6; dil 1:30; Thermo Fisher Scientific, UK), CD20 (L26; dil 1:200; Dako, Denmark), MUM-1 (MUM1p; dil 1:25; Dako, Denmark), Ki-67 (MIB-1; dil 1:75; Dako, Denmark), C-MYC (Y69; dil 1:500; Ventana, Arizona), BCL2 (100/D5; dil 1:50; Thermo Fisher Scientific, UK), BCL6 Ab2 (BL6.02; dil 1:20; Thermo Fisher Scientific, UK), Anti-STAT3 (phospho Y705) (1:50, Abcam, USA), Anti-JAK1 (phospho Y1022+ Y1023) (1:50, Abcam, USA), LMP1 (1:100, Dako, Denmark) were then incubated for 15 minutes at room temperature. Enzyme-conjugated secondary antibody was applied

after the slides wash by Bond wash solution (Leica). 3,3′-Diaminobenzidine (DAB) stained slides resulted in brown precipitation and finally counterstained with Hematoxylin. All slides were mounted and viewed under Olympus microscope for scoring under 100X magnification. The immunostaining for immunomarkers is robust and hence, the reproducibility cut-off values were accessed as below: (i) 30% for pSTAT3; (ii) 10% for pJAK1; (iii) 40% for MYC; (iv) 50% for BCL2; (v) 70% for BCL6; (vi) 10% for LMP1.[12-14]

Fluorescence *In Situ* Hybridisation (FISH)

All FFPE tissue was sectioned at 2 μm for interphase FISH assay. *c-MYC* gene was labelled by break-apart probes. FISH DNA split probe and FISH Accessory Kit (Dako, Glostrup, Denmark) were used in this experiment. The method was carried out as suggested by the manufacturer. Briefly, de-paraffinisation and rehydration were carried out prior to the staining procedure. After digestion with pepsin solution for 30 minutes at 37°C in Dako hybridiser. Denaturation and hybridisation with probe *c-MYC* FISH DNA Probe, Split Signal (Dako, Glostrup, Denmark) for the detection of translocations involving the *c-MYC* locus at chromosome 8q24 by FISH. The slides were subsequently mounted and viewed under the Zeiss Axioimager Fluorescent microscope with the Metasystem's ISIS Metafer software at 630X magnificent for scoring and localisation purpose.

Using break-apart probes, a translocation is identified if: (i) one pair of red and green signals is fused while the other pair is two or three signals widths apart, (ii) red and green signals are seen but none of them are fused, (iii) one pair of red and green signals is fused while either red or green signal of the other pair is gone, or (iv) deleted. Translocation must be evident in at least 20% of the tumour cells. No translocation is identified if there are: (i) two pairs of fused red and green signals in a nucleus, and/or (ii) all signals seen within the nucleus are paired and no extra signal is visible. A gain of signals is defined if extra pairs of signals are observed in ≥50% of cells. The cut-off values are based on a controlled study done in the normal tonsil.

Statistical analysis

Results were expressed in means value ± standard deviation (SD). Statistical analysis with Chi-squared and Fisher's exact test to identify between-group differences using IBM SPSS Statistics (version 23; SPSS Inc., Chicago, IL, USA). Statistical significance expressed as ***P <0.001; **P <0.01; *P <0.05.

RESULTS

Among the 51 DLBCL cases, 19.6% (10/51) cases had EBER expression. The median age of EBV(+)-DLBCL patients was 56.0, range from 35-81. Male had higher prevalence which consists of 70%. 60% EBV(+)-DLBCL present in the nodal site. Immunophenotyping of ten cases EBV(+)-DLBCL was listed in Table 1. All cases had CD20+ of B-cell expression. Markers of CD10, BCL6 and MUM1 according to Hans algorithm was used to categorise the 51 DLBCL cases into the germinal centre (GCB) and non-GCB.[15] The presence of EBV determined by EBER-ISH assay (Fig. 1a). Out of ten cases of EBV(+)-DLBCL, 90% were non-GCB subtype (p=0.011), non-GCB subtype consisting of ABC-DLBCL. Besides, to investigate the protein and gene expression in ten cases of EBV(+)-DLBCL,

TABLE 1: Immunophenotyping and genotyping of EBV-positive DLBCL

Cases	Site		Age	Gender	COO	LMP1	Latency type	pJAK1	pSTAT3 (% expression)	MYC (% expression)	*c-MYC* FISH
1	extra-nodal	tonsil	72	M	nGCB	+	II/III	-	-	-	-
2	extra-nodal	lumbar	55	M	GCB	+	II/III	-	-	+ (40)	ND
3	nodal	cervical node	57	M	nGCB	+	II/III	-	+ (50)	-	-
4	extra-nodal	tonsil	39	M	nGCB	ND	ND	+	+ (50)	-	-
5	nodal	lymph node	81	F	nGCB	+	II/III	-	+ (80)	+ (60)	-
6	extra-nodal	nasopharynx	35	F	nGCB	+	II/III	+	ND	+ (90)	ND
7	nodal	neck node	59	M	nGCB	+	II/III	-	-	-	ND
8	nodal	lymph node	55	M	nGCB	+	II/III	+	+ (80)	+ (80)	-
9	nodal	lymph node	53	M	nGCB	-	I	+	+ (60-70)	+ (40)	-
10	nodal	pelvic node	58	F	nGCB	+	II/III	-	+ (35-40)	+ (50)	ND
p-value against EBV	--	--	--	--	0.011*	--	--	0.527	0.678	0.527	--

F=Female, M=Male; COO: cell of origin; GCB: Germinal-centre like; nGCB: non-GCB; ND: Not Detected due to insufficient tissue

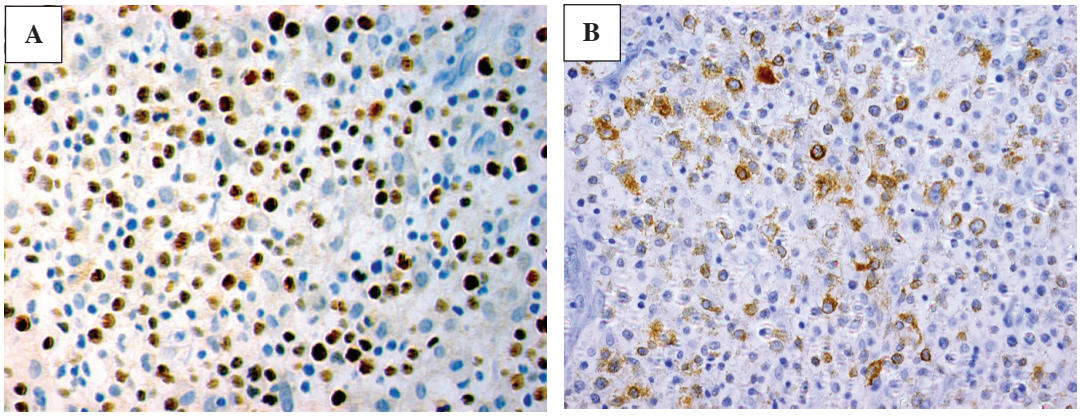

FIG. 1: (A) EBER-ISH Assay Staining of EBER latent gene in nuclear to detect the infected cells. Figure shows >80% expression in tumour nuclear cells. (cut-off: ≥50%). Magnification: 630x. (B) LMP1 Immunophenotyping EBV latent membrane protein localise to cells membrane and cytoplasm. Figure shows >30% expression in tumour cells. (cut-off: ≥10%). Magnification: 400x

IHC for LMP1, pJAK1, pSTAT3 and MYC; FISH assay for *c-MYC* gene rearrangement were carried out. To determine the latency phenotype, LMP1 expression was performed and 88.9% were found to be under latency type II or type III (Fig. 1b).

To investigate the JAK/STAT activity in DLBCL, the presence of pSTAT3(Ty-705) was examined through IHC assay (Fig. 2a). A total of 66.7% (6/9) EBV(+)-DLBCL showed positivity of pSTAT3, majority (5/9) of immunotype showed 50% and above expression. Among the positive expression of pSTAT3, all were non-GCB subtype. pJAK1 expression, which is the up-stream receptor of STAT3 was examined, to elucidate the pathological role of JAK1 in JAK/STAT pathway in pSTAT3(+) cases. In EBV(+)-DLBCL cases, 40% (4/10) had pJAK1 positive expression (Fig. 2c)

To determine the association between pSTAT3 and MYC, FISH and IHC experiments were carried out to observe c-*MYC* gene and protein expression (Fig. 3). None of EBV(+)-DLBCL had *c-MYC* gene rearrangement, while 60% EBV(+)-DLBCL cases showed MYC positive protein expression. No significant association between EBV and MYC protein expression (p=0.527). *c-MYC* rearrangement negatively shown in EBV(+)-DLBCL may indicated that the interference of EBV latent genes have no impact on the *c-MYC* rearrangement in DLBCL.

In EBV(+)-DLBCL, 80% of positive pSTAT3 cases expressed LMP1 (4/5). Although it is difficult to conclude LMP1 and the role of STAT3-mediated pathway has deregulated MYC based on the protein expression, immunophenotyping of clinical cases in this study had successfully shown the high frequency of co-expressions of both LMP1 and pSTAT3. For the upstream receptor of pSTAT3, all pJAK1 showed positive expression in non-GCB subtypes of EBV(+)-DLBCL, but not associated with the EBER (p=0.527) and LMP1 expression (p=0.375) (Data not shown).

Several patterns listed as Pattern I-VI were observed to delineate the MYC expression and pJAK1/pSTAT3 in EBV(+)-DLBCL (Table 2). Among the upregulated MYC protein positive cases, high level of pSTAT3 concurrently expressed (Pattern I and II), of which 80% (4/5) cases had pSTAT3 positivity. For pJAK1 expression, only 40% (2/5) was observed in Pattern II. For MYC protein negative cases (Pattern IV to VI), only 50% (2/4) pSTAT3 was positively expressed in each Pattern IV and V. No activation of JAK1/STAT3 in Pattern III and VI that only consist of one case for each. In a nutshell, pJAK1 was independently expressed, STAT3 was not canonically phosphorylated by JAK1, suggested JAK1 was not imposed on the MYC deregulation in EBV(+)-DLBCL.

DISCUSSION

EBV(+)-DLBCL is predominantly showed with non-GCB immunophenotype and characterised by NF-κB activation,[11,16] whereby our EBV(+)-DLBCL cases were significantly associated with non-GCB subtype (p=0.011). ABC-DLBCL subtype in non-GCB is linked to NF-κB signalling pathways, constitutive activation of NF-κB pathway contributes to the secretion of the IL6 or IL10. The cytokines thereafter stimulate the JAK/STAT pathway and activate the STAT3

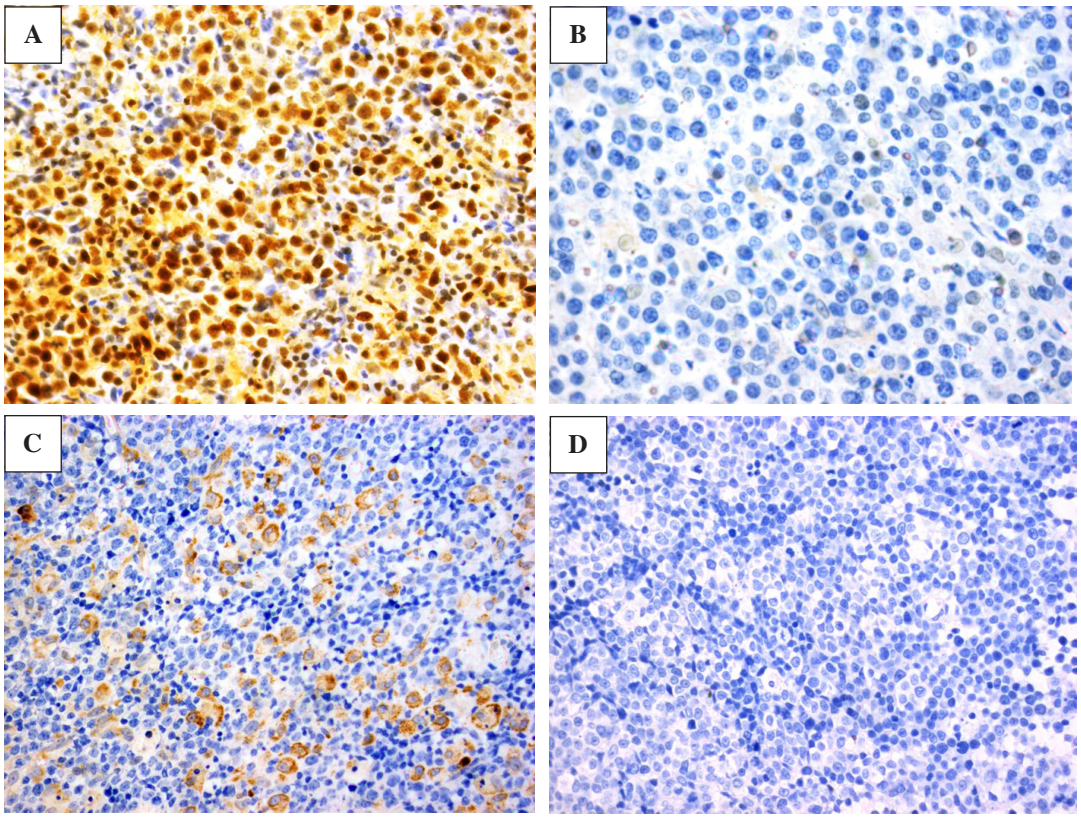

FIG. 2: (A) pSTAT3 Immunophenotyping DAB-staining of pSTAT3, brownish precipitation indicates the pSTAT3 protein expression (cut-off: ≥30%). pSTAT3(+)-EBV(+)-DLBCL with >85% expression. (B) pSTAT3(-)-EBV(+)-DLBCL. (C) pJAK1 Immunophenotyping DAB-staining of pJAK1, brownish precipitation indicates the pJAK1 protein expression (cut-off: ≥10%). pJAK1(+)-EBV(+)-DLBCL with >60% expression. (D) pJAK1(-)-EBV(+)-DLBCL. Magnification: 400x.

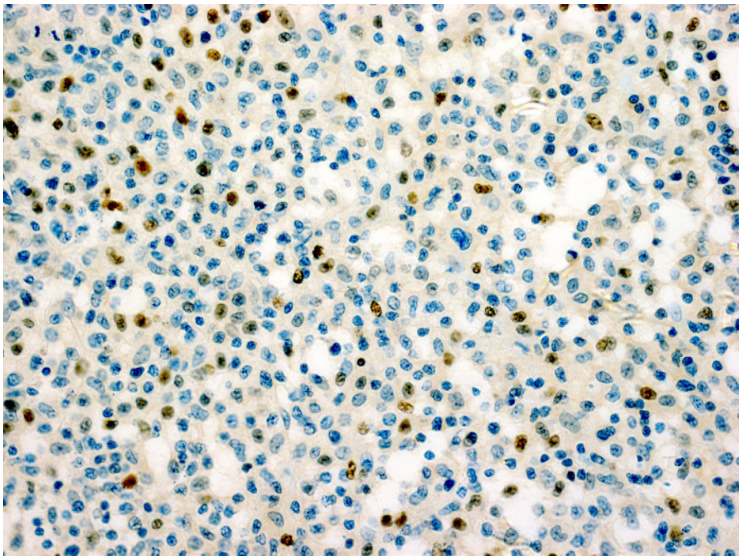

FIG. 3: MYC Immunophenotyping (cut-off: ≥40%) More than 40% tumours cells show the MYC protein expression. The result has been evaluated in FFPE tissue by immunohistochemistry assay, brownish precipitation indicates the expression of MYC protein in tumour cells. Magnification: 400x.

TABLE 2: Expression of MYC, pJAK1 and pSTAT3 in EBV-positive DLBCL

Patterns	MYC Expression	pJAK1 Expression	pSTAT3 Expression	No. of Case	No. of case for MYC and pSTAT3 Expression n (%)	The possible pathway
I	MYC(+)	pJAK1(-)	pSTAT3(+)	2	4 (80)	STAT3-mediated
II	MYC(+)	pJAK1(+)	pSTAT3(+)	2		JAK1/STAT3 pathway
III	MYC(+)	pJAK1(-)	pSTAT3(-)	1	1 (20)	No activation of JAK1/STAT3 pathway
IV	MYC(-)	pJAK1(-)	pSTAT3(-)	2	2 (50)	No activation of JAK1/STAT3 pathway
V	MYC(-)	pJAK1(-)	pSTAT3(+)	1	2 (50)	STAT3-mediated
VI	MYC(-)	pJAK1(+)	pSTAT3(+)	1		JAK1/STAT3 pathway

Note: Case 6 was excluded in this table due to insufficient tissue.

proteins. Gene Expression Profiling (GEP) revealed the latent genes of EBV has induced the NF-κB and JAK/STAT pathways, furthermore higher percentage of clinical samples has pSTAT3 protein expression for cases EBV(+)-DLBCL-E and EBV(+)-DLBCL with results 80% and 39% respectively.[10] STAT3 is relevant to the EBNA1-Qp regulation, and the expression of LMP1 is controlled by the JAK/STAT pathway.[17]

pSTAT3 and pJAK1

pSTAT3 expression can be found in both GCB and non-GCB DLBCL, the expression on the DLBCL is dominance to non-GCB subtype, while percentage of pSTAT3 positive cases among GCB and non-GCB subtypes varies. Gupta *et al*. observed 27% and 58% pSTAT3 expression in GCB and non-GCB respectively. Higher frequency was observed by Ding *et al*., which is 70% pSTAT3 expression in non-GCB subtype.[12,18] Furthermore, DLBCL with pSTAT3 expression reported to have inferior event-free survival (EFS). Ok *et al*. observed 16% expression in *de novo* DLBCL and was more associated to ABC subtype with MYC expression.[13] ZL *et al*. (2011) finding also concurred to the association conclusion, and 32.4% with strong nuclear expression has been reported.[19]

66.7% of EBV(+)-DLBCL carried pSTAT3 positivity (Table 1). In Hodgkin's disease tissue, expression of LMP1 is relevant to STAT3 regulation. LMP1 mimics CD40 signal and activate NF-κB pathway, up-regulate IL6 and subsequently induced the JAK/STAT signalling pathway.[20] Constitutive NF-κB pathway is usually activated in ABC-DLBCL, forming CARD11/ BCL10/MALT1 complex and activate IKKβ, results in secretion of IL6 and/or IL10.[21] Cytokines IL6 and/or IL10 induce the phosphorylation of STAT3, results in the homo- or heterodimer formation of STAT3. Of note, the stimulation of IL6 can be regulated by NF-κB or LMP1-mediated and therefore triggered the STAT3 dimerisation.

In contrast to JAK2, JAK1/STAT3 pathway frequently occurred in non-GCB of DLBCL, ABC subtype. ABC-DLBCL of which associated with the constitutive NF-κB pathway been reported.[22-23] Since JAK2 is not activated in the

ABC-DLBCL cells, therefore JAK1 activation was focused in this study. In our hypothesis, the aberration of JAK1/STAT3 pathway translocates to the nucleus, results in up-regulation of MYC expression in DLBCL. Past studies revealed that JAK1 contributes to the cell survival of ABC-DLBCL, regulates IRF4, MYD88 and MYC. A constitutive JAK1/STAT3 signalling pathway is activated in ABC-DLBCL. However, not all JAK1 involved in the STAT3-mediated pathway. JAK1-dependent H3Y41 phosphorylation involves nearly 3,000 genes in chromatin and regulates IRF4, MYD88 and MYC expression in ABC-DLBCL.[24] In EBV(+)-DLBCL, pJAK1 immunophenotyping was not significantly present in DLBCL tumour cells, suggesting that pJAK1 was not involve in LMP1-mediated pathway, consistent with the report by Gires *et al*.[25]

The 88.9% LMP1 positivity rate (Table 1) in EBV(+)-DLBCL highlighted that LMP1 induced the tumour development, which may throw light on the role of EBV genome in STAT3-mediated pathway. For JAK1/STAT3 pathway, the pJAK1 result did not meet the initial hypothesis. Although JAK1 is the upstream regulator of STAT3 in DLBCL non-GCB subtype, STAT3 activation was more likely to be regulated by other receptors in MYC mechanism, which means JAK1 was not imposed on the MYC deregulation. The concurrent expression of pSTAT3 and MYC denote the STAT3-mediated involved in the regulation mechanism.

MYC deregulation

In the ten EBV(+)-DLBCL cases, whether EBV has a role in MYC deregulation mechanism is rather interesting. In the current finding, more than half of the cases (60%, 6/10) had MYC protein expression (Table 1). Interestingly, none of the EBV(+)-DLBCL cases showed *c-MYC* gene rearrangement even though they had MYC positive protein expression. It was speculated that MYC deregulation is not initiated or limited from the *c-MYC* gene, but rather complex events or an alternative transcriptional mechanism may have occurred.

Deregulation of MYC can be caused by several mechanisms, both directly or indirectly. Three novel mechanisms for activation of *c-MYC* genes have been illustrated which are insertional mutagenesis, chromosomal translocation and gene amplification.[26] The deregulation of MYC can either be initiated by the genes alterations or independently controlled by the activation of hormones and growth factors, their receptors, second messengers and transcriptional effectors. MYC expression is the hallmark of BL, performed simple karyotype with *Ig-MYC* rearrangement, whereas in high-grade B-cell lymphoma, or intermediate BL/DLBCL have complex karyotype with Ig light chain or non-*Ig-MYC* rearrangement such as *BCL6, BCL11A*, *PAX5* or *ICAROS49*.[27-28] Both BL and high grade B-cell lymphoma have a very aggressive manner, with proliferation marker Ki-67 >90%. EBV latency type III program, TLR9 and CD40 in the NF-κB pathway were the three different inducers for the upregulation of MYC. Deregulation of MYC in NF-κB activated B cell by the three inducers would constantly increase the proliferation rate and aggressiveness of tumour cells. Through CD40 activation, the deregulation of MYC transformed indolent lymphoma to aggressive status. *c-MYC* is an NF-κB co-transforming event in aggressive ABC-DLBCL.[29] In this study, strong and concurrently expression of pSTAT3 and MYC indicated instead of *c-MYC* rearrangement, pSTAT3-mediated is the transcription factor of MYC deregulation in EBV(+)-DLBCL (Fig. 4).

MYC is the hallmark of BL, however, recent studies had been reported not all BL with MYC protein expression has *c-MYC* gene rearrangement. EBV microRNA (miRNAs) might be an alternative agent to involve in the deregulation of MYC. EBV expressed 25 miRNAs and is involved in the viral pathogenesis and lymphogenesis. EBV miRNAs target cellular transcripts during latent infection thereby regulates the host environment. The miRNA can be targeted by host *c-MYC* miRNA and results in MYC deregulation. Via numerous miRNA, an oncomiR, miR-155 is induced by LMP1 in NF-κB pathway and results in cells survival.[30-32] In this study, no *c-MYC* gene rearrangement occurred in EBV(+)-DLBCL. Therefore, EBV miRNA might potentially play a role in the pathogenesis of the malignancies. More studies about the EBV miRNAs are needed to explore the association of EBV and MYC expression in the possibilities of the neoplasia.

In conclusion, none of the EBV(+)-DLBCL cases showed *c-MYC* gene rearrangement, whereas 60% of EBV(+)-DLBCL had MYC protein expression, implying that a direct event that initiated by *c-MYC* gene did not occur in the MYC deregulation, an alternative mechanism such as secondary or tertiary event has emerged in EBV(+)-DLBCL. Furthermore, MYC protein

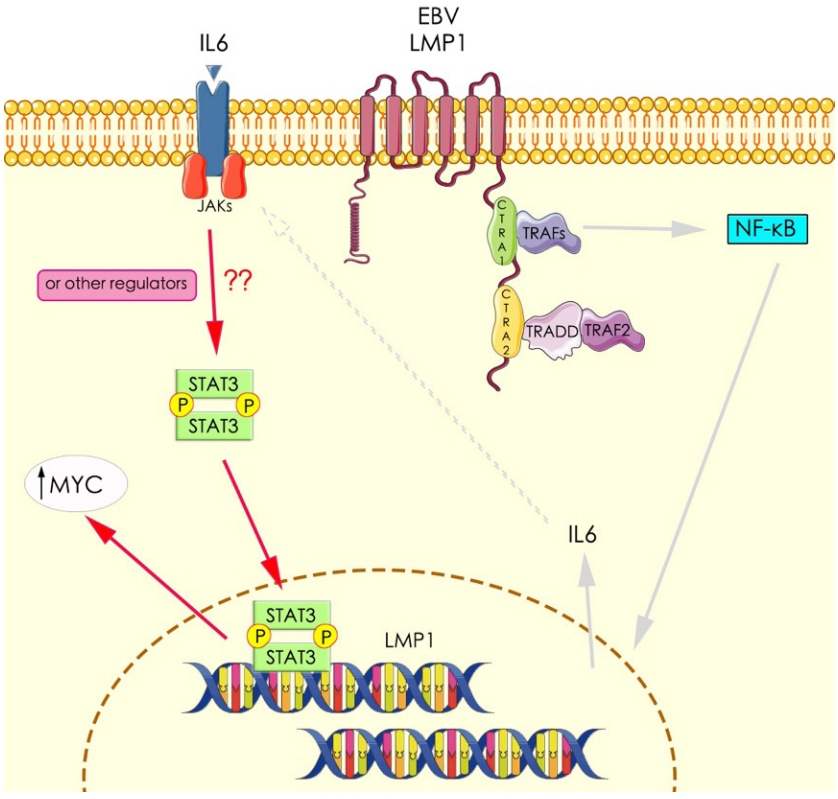

FIG. 4: Expression of EBV, pJAK1/pSTAT3 and MYC. JAK/STAT is cytokines (IL2, IL6 and etc.) and hormone regulatory pathway. (Grey arrows) LMP1 mimic CD40 signal and results in NF-κB signalling pathway. Constitutive NF-κB eventually induce IL6 and stimulate JAK/STAT pathway. In other words, a cross-talk mechanism is occurred, LMP1 triggered the signalling pathway and activates STAT3. Red arrows indicates our hypothesis) STAT3 up-regulated MYC protein expression in EBV(+)-DLBCL. Besides JAK1, we speculated other regulators involved in this mechanism.

expression with a frequency of 80% also concurrently expressed pSTAT3 (Pattern I and II) implies that MYC was up-regulated by STAT3 regulatory (Table 2). From the expression of pJAK1, not all STAT3 was activated by JAK1, implied that others tyrosine kinase or protein regulators may be involved. The result points that STAT3 was the key protein in the regulation, therefore, we suggested pSTAT3 is a potential target for therapeutic agents in EBV(+)-DLBCL.

In EBV(+)-DLBCL, there is no association between LMP1 and pSTAT3 although LMP1 is frequently observed in the EBV(+)-DLBCL with pSTAT3 positivity (80%, 4/5), co-expression of pSTAT3 and MYC indicated that MYC protein was up-regulated by STAT3 in EBV(+)-DLBCL cases. However, pJAK1 and pSTAT3 not always concurrently expressed. Therefore, instead of JAK1/STAT3 signalling, it is suggested that STAT3-mediated pathway is involved in MYC deregulation in EBV(+)-DLBCL and the viral infection or its latent protein LMP1 may play a role in the MYC deregulation mechanism.

ACKNOWLEDGEMENTS

We thank Pantai Premier Pathology Lab, Kuala Lumpur, Malaysia for providing tissue blocks.

Funding: This study was supported by *Fundamental Research Grant Scheme* from Ministry of Education, Malaysia (FRGS/1/2014/SKK06/UCSI/01/1).

Conflict of interest: Nothing to declare, no potential conflicting interests.

Compliance with Ethical Standards: This study was approved by ethics committee of Faculty of medicine and Health Sciences, UCSI University, Malaysia (Proj-FMHS-EC-2014-009).

REFERENCES

1. Lu TX, Liang JH, Miao Y, *et al*. Epstein-Barr virus positive diffuse large B-cell lymphoma predict poor outcome, regardless of the age. Sci Rep. 2015; 5: 12168.

2. Castillo JJ, Beltran BE, Miranda RN, Paydas S, Winer ES, Butera JN. Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly: What we know so far. Oncologist. 2011; 16(1): 87-96.
3. Ok C, Ye Q, Li L, *et al*. Age cutoff for Epstein-Barr virus-positive diffuse large B-cell lymphoma–is it necessary? Oncotarget. 2015; 6(16): 13933-45.
4. Niitsu N, Okamoto M, Miura I, Hirano M. Clinical significance of 8q24/*c MYC* translocation in diffuse large B-cell lymphoma. Cancer Sci. 2009; 100(2): 233-7.
5. Ladanyi M, Offit K, Parsa N, *et al*. Follicular lymphoma with t(8;14)(q24;q32): a distinct clinical and molecular subset of t(8;14)-bearing lymphomas. Blood. 1992; 79(8): 2124-30.
6. Offit K, Wong G, Filippa DA, Tao Y, Chaganti RS. Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: clinical correlations. Blood. 1991; 77(7): 1508-15.
7. Lu TX, Liang JH, Miao Y, *et al*. Epstein-Barr virus-positive diffuse large b-cell lymphoma predict poor outcome, regardless of age. Hemato Oncol. 2015; 33(S1): 244-321.
8. Bowman T, Broome MA, Sinibaldi D, *et al*. Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. Proc. Natl. Acad. Sci. U.S.A. 2001; 98(13): 7319-24.
9. Avalle L, Pensa S, Regis G, Novelli F, Poli V. STAT1 and STAT3 in tumorigenesis: A matter of balance. JAKSTAT. 2012; 1(2): 65-72.
10. Kato H, Karube K, Yamamoto K, *et al*. Gene expression profiling of Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly reveals alterations of characteristic oncogenetic pathways. Cancer Sci. 2014; 105(5): 537-44.
11. Swerdlow SH, Campo E, Pileri SA, *et al*. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood. 2016; 127(20): 2375-91.
12. Gupta M, Maurer MJ, Wellik LE, *et al*. Expression of Myc, but not pSTAT3, is an adverse prognostic factor for diffuse large B-cell lymphoma treated with epratuzumab/R-CHOP. Blood. 2012; 120(22): 4400-6.
13. Ok CY, Chen J, Xu-Monette ZY, *et al*. Clinical implications of phosphorylated STAT3 expression in De Novo diffuse large B-cell lymphoma. Clin Cancer Res. 2014; 20(19): 5113-23
14. Nowakowski GS, Czuczman MS. ABC, GCB, and double-hit diffuse large B-cell lymphoma: Does subtype make a difference in therapy selection? Am Soc Clin Oncol Educ Book. 2015; 35: e449-57.
15. Perry AM, Mitrovic Z, Chan WC. Biological prognostic markers in diffuse large B-cell lymphoma. Cancer Control. 2012; 19(3): 214-26.
16. Montes-Moreno S, Odqvist L, Diaz-Perez JA, *et al.* EBV-positive diffuse large B-cell lymphoma of the elderly is an aggressive post-germinal center B-cell neoplasm characterized by prominent nuclear factor-kB activation. Mod Pathol. 2012; *25*(7): 968-82.
17. Chen H, Lee JM, Wang Y, Huang DP, Ambinder RF, Hayward SD. The Epstein-Barr virus latency BamHI-Q promoter is positively regulated by STATs and Zta interference with JAK/STAT activation leads to loss of BamHI-Q promoter activity. Proc Natl Acad Sci USA. 1999; 96(16): 9339-44.
18. Ding BB, Yu, JJ, Yu RY, *et al.* Constitutively activated STAT3 promotes cell proliferation and survival in the activated B-cell subtype of diffuse large B-cell lymphomas. Blood. 2008; 111(3): 1515-23.
19. Wu ZL, Song Y, Shi Y, Zhu J. High nuclear expression of STAT3 is associated with unfavorable prognosis in diffuse large B-cell lymphoma. J Hemato Oncol. 2011; 4(1): 31.
20. Rui L, Emre NC, Kruhlak MJ, *et al*. Cooperative epigenetic modulation by cancer amplicon genes. Cancer Cell. 2010; 18(6): 590-605
21. Chen H, Lee JM, Zong Y, *et al*. Linkage between STAT regulation and Epstein-Barr virus gene expression in tumors. J Virol. 2001; 75(6): 2929-37.
22. Ziarkiewicz M, Wołosz D, Dzieciątkowski T, *et al*. Epstein–Barr virus-positive diffuse large B cell lymphoma in the experience of a tertiary medical center in Poland. Arch Immunol Ther Exp (Warsz). 2016; 64(2): 159-69.
23. Zhu F, Hwang B, Miyamoto S, Rui L. Nuclear import of JAK1 is mediated by a classical NLS and is required for survival of diffuse large B-cell lymphoma. Mol Cancer Res. 2017; 15(3): 348-57.
24. Rui L, Drennan AC, Ceribelli M, *et al*. Epigenetic gene regulation by Janus kinase 1 in diffuse large B-cell lymphoma. Proc. Natl. Acad. Sci. U.S.A. 2016; 113(46): E7260-E7267.
25. Gires O, Kohlhuber F, Kilger E, *et al*. Latent membrane protein 1 of Epstein–Barr virus interacts with JAK3 and activates STAT proteins. EMBO J. 1999; 18(11): 3064-73.
26. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer. 2008; 8(12): 976-90.
27. Bellan C, Lazzi, S, Hummel M, *et al*. Immunoglobulin gene analysis reveals 2 distinct cells of origin for EBV-positive and EBV-negative Burkitt lymphomas. Blood. 2005; 106(3): 1031-6.
28. Cai Q, Medeiros LJ, Xu X, Young KH. MYC-driven aggressive B-cell lymphomas: biology, entity, differential diagnosis and clinical management. Oncotarget. 2015; 6(36): 38591-616.
29. David A, Arnaud N, Fradet M, *et al*. c-Myc dysregulation is a co-transforming event for nuclear factor-κB activated B cells. Haematologica. 2017; 102(5): 883-94.
30. Skalsky RL, Corcoran DL, Gottwein E, *et al*. The viral and cellular microRNA targetome in lymphoblastoid cell lines. PLoS Pathog. 2012; 8(1): e1002484.
31. Gatto G, Rossi A, Rossi D, Kroening S, Bonatti S, Mallardo M. Epstein–Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-κB pathway. Nucleic Acids Res. 2008; 36(20): 6608-19.
32. Ok CY, Li L, Young KH. EBV-driven B-cell lymphoproliferative disorders: from biology, classification and differential diagnosis to clinical management. Exp Mol Med. 2015; 47(1): e132.