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Establishing etiopathogenesis in Epstein-Barr virus associated malignancies using chromogenic *in situ* hybridization

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ABSTRACT

Background: Epstein–Barr virus is a human herpes virus ubiquitously infecting 90% of world population and causing 1% of tumors worldwide. The objectives were to establish etiopathogenesis in the Epstein-Barr virus (EBV) associated malignancies reported in our laboratory using the gold standard test, i.e., chromogenic *in situ* hybridization (CISH) for Epstein-Barr virus - encoded RNA (EBER). **Aims and Objectives:** We performed CISH on 244 cases of associated malignancies in the year 2013–2016, the decision was taken after H and E diagnosis and immunohistochemistry confirmation. **Observation and Results:** In the period of 2013–2016, 253 cases were evaluated by CISH for EBER. The age range in our study was 5–88 years with the mean age of 41.8 years, a slight male predominance was noted with male: female ratio of 1.5:1. CISH in 61 Classical Hodgkin lymphoma cases of 90cases were positive **Conclusion**: EBER ISH is the single best histochemical assay for defining EBV - related neoplasia. It is likely that emerging technologies such as gene expression profiling and proteomics will identify patterns of viral and human gene expression correlating with diagnosis, prognosis, and outcome in response to therapy.

Key words: Chromogenic *in situ* hybridization, Epstein-Barr virus, Hodgkin's lymphoma, Nasopharyngeal carcinoma

Introduction

Epstein–Barr virus is a human herpes virus ubiquitously infecting 90% of world population and causing 1% of tumors worldwide, due to which WHO has classified it as a tumor virus.[1] Epstein-Barr virus (EBV) is the epitome of B lymphotropic virus as the disease spectrum extends from self-limited infectious mononucleosis to Hodgkin's lymphoma, T/NK cell malignancies and smooth muscle tumor.[2] The characteristic EBV gene Type 0 latency (Epstein-Barr virus - encoded RNA [EBERs], BamHI-A rightward transcripts [BARTs]) in AIDS-related plasmacytic lymphomas and circulating B-cells, Type I (Epstein-Barr nuclear antigen 1 [EBNA1], latent membrane protein 2 [LMP2], EBERs, and BARTs) is expressed in Burkitts lymphoma, Type II (EBNA1,2,3A,3B,3C, LMP1, LMP2, EBERs, LMP1, LMP2, EBERs, and BARTs) in AIDS-related Burkitts or primary effusion lymphoma, peripheral T-cell lymphoma, NK/T-cell lymphoma-nasal type, nasopharyngeal carcinoma, and gastric adenocarcinoma, Type III in AIDS-related immunoblastic or brain lymphoma, infectious mononucleosis, chronic active EBV infection, lymphoblastoid cell lines *in vitro* and in tonsillar B-cells.[3] Accurate laboratory tests to detect EBV are needed for epidemiological and clinical management. EBER *in situ* hybridization (ISH) has been recommended as the best test for detecting and localizing latent EBV in tissue samples when more than 1 million copies per cell. The identification of EBV implies that the patient is candidate for laboratory monitoring of tumor burden based on the molecular assay for EBV viral load and treatment using EBV-targeted oncologic therapies.[3]

Aim and Objectives

As a robust National Reference Laboratory with a significant number of malignant cases including lymphoma, we decided to analyze EBER transcripts in the reported associated malignancies. The objective was to establish etiopathogenesis in the EBV-associated malignancies reported in our laboratory using the gold standard test, i.e. chromogenic ISH (CISH) for EBER.

Methods

We performed CISH on 244 cases of associated malignancies in the years 2013–2016; the decision was taken after H and E diagnosis and immunohistochemistry (IHC) confirmation. The test was performed on formalin-fixed, paraffin-embedded - formalin fixed paraffin embedded tissue. A single representative block was chosen from each case. It was the same block on which IHC had been performed. EBER by ISH is detected by DNA probes. The hybridization results in the duplex formation of digoxigenin-labeled probe with the EBV RNA in the test material which is indirectly detected using enzyme-conjugated antibodies directed against digoxigenin or unconjugated antibodies detected by a secondary polymerized enzyme-conjugated antibody. The enzymatic reaction of a chromogenic substrate leads to the formation of a color

precipitate in the nucleus of these cells that is visualized by light microscopy. The negative slide was run in parallel, the U6 control RNA-targeted in this study was localized to the nucleus of all types of cells. The positive control section was processed on the same slide with the test section. The analysis of the positive smears was performed according to Guidelines for interpreting EBER ISH one which states that.

- The morphologist must be competent in distinguishing read Sternberg/Hodgkin (RS/H) cells from non-tumor cells. The cytologic features and distribution of RS/H cells should be assessed on matched H and E - stained sections before interpreting EBER.
- To interpret a case as positive, the EBER single must be unequivocally localized to RS/H cells. The fraction of RS/H cells expressing EBER varies among cases, with most cases having a high fraction of positive tumor cells. For purposes of identifying all Epstein-Barr virus-related Hodgkin cases, just one unequivocal RS/H cell a cases positive, equivocal cases frequently are resolved when EBER and H and E stains are evaluated in parallel.
- The EBER single is localized to the nucleus, sometimes spearing or rimming the nucleus. A negative EBER result can be interpreted as negative only if RNA is shown to be preserved and available for hybridization in tumor cells. A variable proportion of background lymphocytes express EBER (usually 0–1%), and these must be distinguished from RS/H cells.

Results

In the period of 2013–2016, 253 cases were evaluated by CISH for EBER. The age range in our study was 5–88 years. With the mean age of 41.8 years; a slight male predominance was noted with male: female ratio of 1.5:1. 95 (37.5%) of the 253 cases were positive. The morphological spectrum examined along with their CISH testing is depicted in Table 1.

From the above table, we can make the following observations that CISH in 90 classical Hodgkin lymphoma cases. 61 (62%) were positive for EBER (Figure. 1). Nasopharyngeal carcinoma (NPC) (6 cases), 14 out of 28 metastatic malignancies were secondary to NPC which were EBER positive, 6 out of 15 Burkitt lymphoma were also EBER positive. Distribution of EBER-positive cases was as follows Figure 1.

Discussion

In recent 2016 WHO classification of lymphoid, histiocytic, and dendritic neoplasms have included EBV-associated disorders such as EBV + diffuse large B-cell lymphoma, not otherwise specified; EBV+ mucocutaneous ulcer which is a newly recognized entity; systemic EBV$^+$ T-cell lymphoma of childhood; Hydroa vacciniforme - like lymphoproliferative disorder which is name changed from lymphoma to lymphoproliferative disorder due to its relationship with chronic active EBV infection and a spectrum in terms of its clinical course.

Available detection methods for EBV are polymerase chain reaction (PCR), ISH, and IHC. ISH is the standard procedure for detecting EBERs. According to some authors, PCR and CISH are equally sensitive in detecting EBV in routinely processed biopsies, while IHC is an insensitive method.[4] EBER 1 and 2 are non-polyadenylated, uncapped, noncoding RNAs of 167 and 172 nucleotides, respectively, and are expressed abundantly in nearly all EBV-infected cells.

U6 control hybridization confirms RNA preservation in the tissue as evidenced by U6 expression in the nuclei of reactive and malignant cells alike. Before concluding that a slide is EBER-negative, it is essential to evaluate a control slide, run in parallel, demonstrating that RNA is present and available for hybridization in the cells of interest. The guidelines which are followed for tissue biopsies are follows;

EBER-ISH assay localizes EBV in biopsy tissue[1]

- A biopsy assessed by EBER-ISH is needed to confirm each diagnosis and its relation to EBV
- Exception of suspected infectious mononucleosis for which clinical findings and serology are usually diagnostic
- Biopsy may be counterproductive because of histological overlap with Hodgkin and non-Hodgkin lymphoma
- Another exceptional lesion is oral hairy leukoplakia in which EBER is down-regulated, whereas lytic proteins BZLF1 and BMRF1 are localized to ballooned cells in mid-layers of the hyperplastic stratified squamous epithelium
- Nearly all undifferentiated NPCs are EBV-related, whereas a lesser proportion of keratinizing NPCs harbor EBV as demonstrated by EBER-ISH.
- Enlarged cervical lymph node representing metastatic spread; the identification of EBER-expressing carcinoma or in a posterior retropharyngeal cervical node should trigger endoscopic examination of the nasopharynx in search of the primary site.
- In a left supraclavicular lymph node, EBER-positive undifferentiated carcinoma or adenocarcinoma should prompt consideration of a gastric primary, since about 7% of gastric carcinomas are infected.

We do IHC for EBV and recommend best practice of doing both in positive case so as to increase detection and association as the sensitivity and specificity in our study were 91% and 94%, respectively, which matched with the results demonstrated by Qi *et al*.[5] which states to perform at least two methods to be performed for accurate results it also states that PCR is the most sensitive of the three methods, but it is unable to provide specific information with regard to the localization of EBV-positive cells which is possible in EBER and EBV by IHC.

The positive cases in our study were either of Classical Hodgkin lymphoma or nasopharyngeal carcinoma. As early as 1966 MacMahon[6] proposed that Hodgkin's disease might be caused by an infectious agent. The first evidence that this

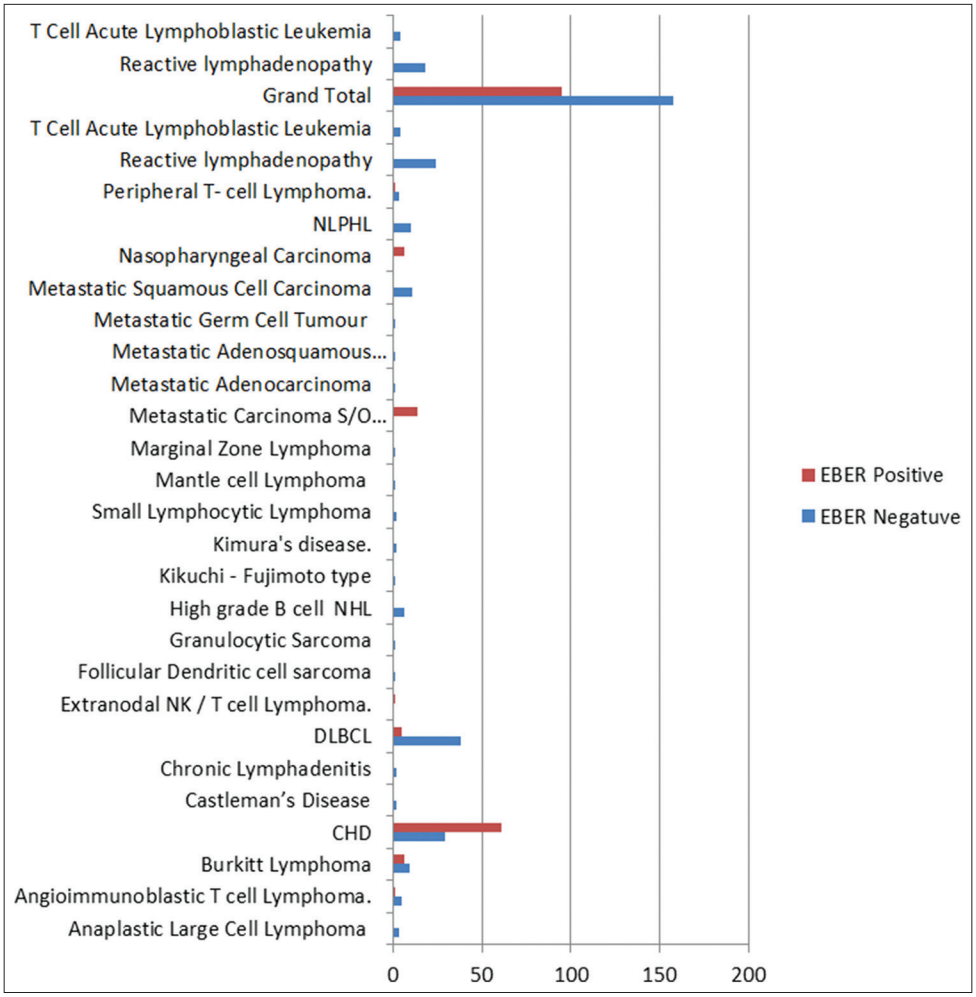

Figure 1: Graphic presentation-EBER CISH Analysis (2013-16)

agent might be EBV was provided by the detection of raised antibody titers to EBV antigens in patients with Hodgkin's disease.[7]

With the advent of cloned viral probes and Southern blot hybridization methods, EBV DNA was initially detected in 20–25% of Hodgkin's disease tumor specimens.[8] However, this approach could not determine the locality of the EBV genome in tissues.

ISH methods to detect EBV DNA provided the first demonstration of its existence in the HRS cells. Subsequently, the demonstration of the abundant EBV early RNA (EBER1 and EBER2) sequences in HRS cells provided a sensitive method for detecting latent infection *in situ*. This technique is generally accepted as the "gold standard" for the detection of latent EBV infection in clinical samples.[9]

In Hodgkin's disease, the bulk of viral genomes is found in monoclonal form, indicating that infection of the tumor cells has occurred before their clonal expansion.[10]

EBV appears to persist throughout the course of Hodgkin's disease and is also found in multiple sites of Hodgkin's disease.[11] In our study also we were able to demonstrate EBER in nodal and extranodal locations. EBV has been detected in the malignant RS/H cells in approximately 40% of patients with Hodgkin disease.[1]

Of the four major histopathological subtypes of Hodgkin lymphoma, the mixed cellularity subtype is most frequently EBV-associated (70%), followed by lymphocyte depletion (50%), nodular sclerosis (20%), and lymphocyte predominant subtypes (<5%).[1] EBV is more commonly associated with the MC subtype and less frequently with the other forms of this disease.[12] We noticed a similar incidence with 62% for EBER. Various studies are shown that EBV-positive rates are higher in male patients than in female patients.[13] We noticed a slight male preponderance as well.

From a clinical standpoint, tests for EBV can be used to help establish a correct diagnosis in patients whose histologic lesion has overlapping features of Hodgkin lymphoma, anaplastic large cell lymphoma, or reactive lymphoid hyperplasia. This was useful in 2 of our cases.

The second most common association in our study was with nasopharyngeal carcinoma, the etiology of which is

Table 1: EBER CISH analysis (2013–2016)

Diagnosis	EBER by CISH		Grand total
	Negative	Positive	
Anaplastic large cell lymphoma	3		3
Angioimmunoblastic T-cell lymphoma	5	1	6
Burkitt lymphoma	9	6	15
CHD	29	61	90
Castleman's disease	2		2
Chronic lymphadenitis	2		2
DLBCL	38	5	43
Extranodal NK/T-cell lymphoma		1	1
Follicular dendritic cell sarcoma	1		1
Granulocytic SARCOMA	1		1
High-grade B-cell NHL	6		6
Kikuchi - Fujimoto type	1		1
Kimura's disease	2		2
Small lymphocytic lymphoma	2		2
Mantle cell lymphoma	1		1
Marginal zone lymphoma	1		1
Metastatic carcinoma S/O nasopharyngeal primary	1	14	15
Metastatic adenocarcinoma	1		1
Metastatic adenosquamous carcinoma	1		1
Metastatic germ cell tumor	1		1
Metastatic squamous cell carcinoma	11		11
Nasopharyngeal carcinoma		6	6
NLPHL	10		10
Peripheral T-cell lymphoma	3	1	4
Reactive lymphadenopathy	24		24
T-cell acute lymphoblastic leukemia	4		4
Grand total	158	95	253

CISH: Chromogenic *in situ* hybridization, EBER: Epstein-Barr virus - encoded RNA, DLBCL: Diffuse large B-cell lymphoma

multifactorial and includes genetic susceptibility, exposure to carcinogens, and prior infection with the EBV. The initial link of EBV infection to NPC was the discovery of elevated immunoglobulins (IgG) and IgA antibody titers to viral-capsid antigen and EA in patients with NPC. The titers correlated with tumor burden, remission, and recurrence and preceded tumor development by 1–2 years, suggesting that reactivation or replication of EBV may be involved in tumorigenesis.[14] In addition, multiple copies of circular EBV DNA and other footprints of the virus are regularly found in carcinoma cells of virtually all low-grade or undifferentiated tumors. Although EBV is certainly implicated in the pathogenesis of NPC, the exact mechanism and pathway by which it exerts its effects are unknown.[15] Nevertheless, EBV studies, particularly circulating plasma EBV DNA levels, have shown utility in staging, prognosis, and post-therapeutic monitoring. In addition, the subset of early-stage patients with high plasma EBV DNA levels are at increased risk for distant metastasis and may, therefore, be candidates for more aggressive systemic treatment early on.[16] EBV is one of the best tumor markers yet discovered. EBV viral load testing has been incorporated into the routine care of patients with post-transplant lymphoproliferative disease, nasopharyngeal carcinoma, and AIDS lymphoma of the brain. EBER ISH is the single best histochemical assay for defining EBV-related neoplasia. It is likely that emerging technologies such as gene expression profiling and proteomics will identify patterns of viral and human gene expression correlating with diagnosis, prognosis, and outcome in response to therapy. A coordinated effort by basic scientists and clinical investigators will improve our arsenal of laboratory methods and better define their clinical utility.

References

1. Gulley ML, Glaser SL, Craig FE, Borowitz M, Mann RB, Shema SJ, *et al.* Guidelines for interpreting EBER *in situ* hybridization and LMP1 immunohistochemical tests for detecting Epstein-Barr virus in Hodgkin lymphoma. Am J Clin Pathol 2002;117:259-67.
2. Delecluse HJ, Feederle R, O'Sullivan B, Taniere P. Epstein Barr virus-associated tumours: An update for the attention of the working pathologist. J Clin Pathol 2007;60:1358-64.
3. Gulley ML, Tang W. Laboratory assays for Epstein-Barr virus-related disease. J Mol Diagn 2008;10:279-92.
4. Hassan R, White LR, Stefanoff CG, de Oliveira DE, Felisbino FE, Klumb CE, *et al.* Epstein-Barr virus (EBV) detection and typing by PCR: A contribution to diagnostic screening of EBV-positive Burkitt's lymphoma. Diagn Pathol 2006;1:17.
5. Qi ZL, Han XQ, Hu J, Wang GH, Gao JW, Wang X, *et al.* Comparison of three methods for the detection of Epstein-Barr virus in Hodgkin's lymphoma in paraffin-embedded tissues. Mol Med Rep 2013;7:89-92.
6. MacMahon B. Epidemiology of Hodgkin's disease. Cancer Res 1996;26:1189-200.
7. Levine PH, Ablashi DV, Berard CW, Carbone PP, Waggoner DE, Malan L. Elevated antibody titers to Epstein-Barr virus in Hodgkin's disease. Cancer 1971;27:416-21.
8. Weiss LM, Strickler JG, Warnke RA, Purtilo DT, Sklar J. Epstein-Barr viral DNA in tissues of Hodgkin's disease. Am J Pathol 1987;129:86-91.
9. Wu TC, Mann RB, Charache P, Hayward SD, Staal S, Lambe BC, *et al.* Detection of EBV gene expression in Reed-Sternberg cells of Hodgkin's disease. Int J Cancer 1990;46:801-4.
10. Herbst H, Niedobitek G. Epstein-Barr virus and Hodgkin's disease. Int J Clin Lab Res 1993;23:13-6.
11. Coates PJ, Slavin G, D'Ardenne AJ. Persistence of Epstein-Barr virus in Reed-Sternberg cells throughout the course of Hodgkin's disease. J Pathol 1991;164:291-7.
12. Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS. Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. Lancet 1991;337:320-2.
13. Glaser SL, Lin RJ, Stewart SL, Ambinder RF, Jarrett RF, Brousset P, *et al.* Epstein-Barr virus-associated Hodgkin's disease: Epidemiologic characteristics in international data. Int J Cancer 1997;70:375-82.

14. Raab-Traub N. Epstein-Barr virus in the pathogenesis of NPC. Semin Cancer Biol 2002;12:431-41.
15. Chan AT, Teo PM, Huang DP. Pathogenesis and treatment of nasopharyngeal carcinoma. Semin Oncol 2004;31:794-801.
16. Chu EA, Wu JM, Tunkel DE, Ishman SL. Nasopharyngeal carcinoma: The role of the Epstein-Barr virus. Medscape J Med 2008;10:165.

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