ESTABLISHING ETIOPATHOGENESIS IN EBV ASSOCIATED MALIGNANCIES USING CHROMOGENIC IN SITU HYBRIDIZATION

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INTRODUCTION

- Epstein-Barr virus (EBV) discovered in 1964 is a ubiquitous human herpes virus that infects more than 90%of the world-wide population & 1% tumors worldwide.[1,2]
- This led the world Health Organization to classify EBV in 1997 as a tumor virus.2
- Epidemiologic, molecular, and immunologic evidence links Hodgkin lymphoma to Epstein-Barr virus (EBV) infection.2
- EBV is the epitome of B lymphotropic viruses, but the spectrum of tumor association extends to T/NK cell malignancies, various type of carcinomas & smooth muscle tumor.[2]
- The entities & the characteristics patterns of EBV gene expression lesional tissues are elucidated in Table 1.[3]

Table 1: Characteristic Patterns of EBV Gene Expression in Normal and Lesional Tissue

Cell or tissue type	Typical EBV gene expression*
AIDS-related plasmablastic lymphoma	Type 0 latency (EBERs, BARTs)
Burkitt lymphoma	Type I latency (EBNA1, LMP2, EBERs, BARTs)
Hodgkin lymphoma	Type II latency (EBNA1, LMP1, LMP2, EBERs, BARTs)
AIDS-related Burkitt or primary effusion lymphoma	Type II
Peripheral T cell lymphoma	Type II
NK/T cell lymphoma, nasal type	Type II
Nasopharyngeal carcinoma	Type II plus BARF1
Gastric adenocarcinoma	Type II plus BARF1
Post-transplant lymphoproliferative disorder	Type III latency (EBNA1, −2, −3A, −3B, −3C; LMP1, LMP2, EBERs, BARTs)
AIDS-related immunoblastic or brain lymphoma	Type III
Infectious mononucleosis	Type III
Chronic active EBV infection	Type III
Lymphoblastoid cell lines in vitro	Type III
Oral hairy leukoplakia	Lytic infection (LMP1, LMP2, BZLF1, BMRF1, BHRF1, BCRF1, and other replication factors)
Remotely infected carriers	
Circulating B cells	Type 0
Tonsil/mucosal B cells	Type II

*Viral gene expression may be focal or variable in a given lesion.

- Accurate laboratory tests to detect EBV are needed for purposes of basic and epidemiologic research and for clinical management.[3]
- EBER in situ hybridization has been recommended as the best test for detecting and localizing latent EBV in tissue samples.[3]
- EBER transcripts are naturally abundant in latently infected cells, with levels often exceeding 1 million copies per cell.[3]
- In addition, identification of tumour associated EBV implies that the patient is a candidate for laboratory monitoring of tumour burden based on molecular assay for EBV viral load & for treatment using EBV targeted oncologic therapies.[3]

AIM

As a robust Indian surgical pathology referral laboratory with a significant number of malignant cases including Lymphomas, we decided to analyze EBER transcripts in the reported associated malignancies.

OBJECTIVE

The objectives was to establish etiopathogenesis in the EBV associated malignancies reported in our laboratory using the gold standard test i.e. CISH for EBER.

MATERIAL & METHODS

- We performed CISH on 28 cases of associated malignancies in the year 2013.
- This decision was taken after H & E diagnosis and IHC confirmation.
- The test was performed on FFPE i.e. 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 In Situ Hybridization Procedure

Principle of the Method[4]

The presence of certain nucleic acid sequences in cells or tissue can be detected by in situ hybridization using labeled DNA probes. The hybridization results in duplex formation of sequences present in the test object with the labeled DNA probe.
Duplex formation of the digoxigenin-labeled probe with Epstein-Barr-Virus (EBV) EBER RNA in the test material is indirectly detected by using enzymeconjugated 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 that is visualized by light microscopy.

Procedure

1. Dewax slides in 3 changes of Xylene.
2. Quenching is carried out for 10 min in 3% H2O2
3. Proteolysis – Put Pepsin solution for 5-10 min at 37 C.
4. Heat Pretreatment Solution EDTA at 95 C.
5. Denature the slides by7 putting CISH probe at 75 C for 5 min.
6. Transfer the slides to a humidity chamber and hybridize for60 min at: 55 C.
7. Remove the coverslip by submerging in 1x Wash Buffer TBS
8. Wash 5 min in 1x Wash Buffer TBS at 55 C 1x Wash Buffer TBS.
9. Apply Mouse-Anti-DIG and incubate for 30 min at 37 C.
10. Wash 3x 1 min in 1x Wash Buffer TBS.
11. Apply Anti-Mouse-HRP-Polymer drop wise for 30 min at 37 C.
12. Wash 3x 1 min in 1x Wash Buffer TBS
13. Apply DAB Solution and incubate for 10-20 min at RT
14. Transfer slides into a staining jar and wash 2 min in running tap water
15. Counterstain 2-5 min with Nuclear Blue/Hematoxylin Solution. .
16. Transfer slides into a staining jar and wash 2 min in running tap water
17. Dehydrate, clear & mount.

Controls

- A negative control slide was run in parallel, demonstrating that RNA is present and available for hybridization in the cells of interest. The U6 control RNA targeted in this study was localized to the nucleus of all types of cells.
- We also run a positive control section & test section on the same slide.

EBER In Situ Hybridization Interpretation

- The pathologist interpreted the slide as positive if chromogen was localized to *the nucleus* of these cells.
- No cytoplasmic signal was labeled as positive.
- Negative staining was defined as the absence of chromogen above background levels in the cells of interest.
- The positive & negative controls of the run were satisfactory.

RESULTS

- In the period of January to December 2013, **28 cases** were evaluated by CISH for EBER.
- The age range in our study was *5 to 74 yrs* with the mean age of 40 yrs.
- A male: female ratio of **1.5:1** was noticed
- ***13 (46.4%)*** of the *28 cases* were positive.

The morphological spectrum examined along with their immunohistochemical confirmation & CISH testing is depicted in Table No.2

Table 2: Morphological, Immunohistochemical & CISH analysis

Case no	Age	Sex	Specimen/Site	H&E morphology	Immunohistochemistry	CISH - EBER
1.	69	F	Cervical Lymph Node	Classical Hodgkin Lymphoma (Mixed cellularity)	CD 15 & CD 30 - Positive	Positive
2.	40	M	Nasopharynx	Nasopharyngeal Carcinoma	CK 5/6 & p63 - Positive	Positive
3.	17	F	Endobronchial biopsy	Classical HL(Mixed cellularity)	CD 15 & CD 30 - Positive	Positive
4.	14	M	Cervical Lymph Node	Classical Hodgkin Lymphoma (Mixed cellularity)	CD 15 : Negative. CD 30 - Positive	Positive
5.	35	M	Axillary Lymph Node	T cell NHL	CD3 & CD43,CD 4 Positive	Negative
6.	47	F	Cervical Lymph Node	Classical Hodgkin Lymphoma (Mixed cellularity) in a treated c/o Marginal zone Lymphoma	CD 15 & CD30 - Positive	Negative
7.	25	M	Cutaneous	Extramedullary plasmablastic Lymphoma	LCA, CD 138, Mum1 – Positive.	Negative
8.	68	F	Nasal	High grade DLBCL	CD 20, LCA Bcl-2 : Positive	Negative
9.	63	F	Axillary Lymph Node	D/D – Reactive lymph node V/s Hodgkin Lymphoma	CD 15 & CD 30 - Negative	Negative
10.	28	M	Cervical Lymph Node	D/D – Reactive lymph node V/s Hodgkin Lymphoma	CD 15 & CD 30 : Negative	Negative
11.	51	M	Cervical Lymph Node	Classical Hodgkin Lymphoma (Mixed cellularity)	CD 15 & CD 30 - Positive	Positive
12.	14	F	Adenoid	Sporadic Burkitt Lymphoma	CD 20, CD 10 , BCL-6 - Positive	Negative
13.	50	M	Axillary Lymph Node	Classical Hodgkin Lymphoma (Mixed cellularity)	CD 15 & CD 30 - Positive	Negative
14.	40	F	Cervical Lymph Node	Classical Hodgkin Lymphoma Nodular sclerosis (NS)	CD 15 & CD 30 - Positive	Negative
15.	52	M	Mesenteric Lymph Node	Plasmablastic Lymphoma	LCA ,CD 138 , CD 43 - Positive	Negative
16.	62	M	Posterior Pharyngeal mass	Poorly differentiated SCC.	P63 & Pan CK - Positive	Negative
17.	43	F	Lower Lid	B cell NHL	CD 20 - Positive	Negative
18.	74	F	Cervical Lymph Node	Classical Hodgkin Lymphoma Mixed cellularity	CD 15 & CD 30 - Positive	Positive
19.	22	M	Cervical Lymph Node	Classical Hodgkin Lymphoma Mixed cellularity	CD15 & CD 30 - Positive	Negative
20.	68	F	Axillary Lymph Node	DLBCL	CD 20 , BCL-2 - Positive	Negative
21.	46	M	Nasopharynx	Nasopharyngeal Carcinoma	Pan-CK & p63 - Positive	Positive
22.	65	M	Cervical Lymph Node	Classical Hodgkin Lymphoma Mixed cellularity	CD 15 & CD 30 - Positive	Positive
23.	46	M	Nasopharynx	Nasopharyngeal carcinoma	Pan CK -Positive	Positive
24.	05	M	Cervical Lymph Node	Classical Hodgkin Lymphoma Nodular Sclerosis	CD 15 & CD 30 : Positive	Positive
25.	18	M	Mediastinal Mass	Relapse in a k/c/o HL-Lymphocyte depleted	CD 30 Positive, CD 15 - Negative	Positive
26.	22	F	Cervical Lymph Node	Classical Hodgkin Lymphoma - Mixed cellularity	CD15 & CD30 -Positive	Negative
27.	17	M	Cervical Lymph Node	Classical Hodgkin Lymphoma - Mixed cellularity	CD15 & CD30 - Positive	Positive
28.	38	M	Axillary Lymph Node	Classical Hodgkin Lymphoma - Mixed cellularity	CD 15 & CD30 - Positive	Positive

Figure 1. Clinical & Morphological Distribution of EBV associated cases analysed by CISH in the present study

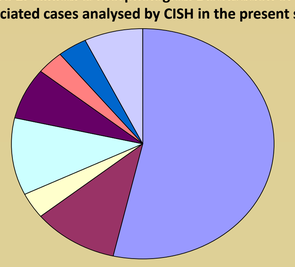
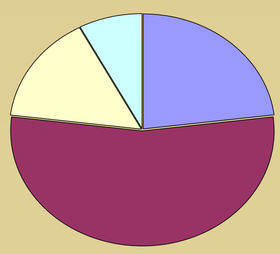


Figure 2. Distribution of EBER positive cases



DISCUSSION

Available detection methods for EBV are: PCR, in situ hybridization (ISH) and IHC.
ISH is the standard procedure for detecting EBV-encoded RNAs (EBERs).
According to some authors, PCR and chromogenic in situ hybridization (CISH) are equally sensitive in detecting EBV , while IHC is an insensitive.
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.[6]
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.[1]

Table 3 : EBV – Associated Diseases with incidence [5]

Diseases	EBV-related (% cases)
Benign reactive infections	
Infectious mononucleosis	>99
Oral hairy leukoplakia	>95
EBV-related hemophagocytic syndrome	100*
Chronic active EBV infection	100*
Hodgkin lymphoma	
Hodgkin lymphoma, all subtypes	40
Hodgkin lymphoma, mixed cellularity	70
Hodgkin lymphoma, nodular sclerosis	20
Hodgkin lymphoma, lymph. Predominant	<5%
Hodgkin lymphoma, lymphocyte depleted	50
Hodgkin lymphoma, AIDS-related	>95
Carcinomas and soft tissue sarcomas	
Nasopharyngeal carcinoma, Asian	>95
Nasopharyngeal carcinoma, USA	75
Lymphoepithelioma-like carcinoma	Most
Gastric adenocarcinoma	7
Smooth muscle tumour in AIDS/transplant	>95
Follicular dendritic cell tumor, IP-like	Most
Non-Hodgkin lymphomas and related neoplasms	
Non-Hodgkin lymphomas, all subtypes	5
Non-Hodgkin lymphomas, diffuse large B cell subtype	15
Richter syndrome (transformed lymphoma)	15
Non-Hodgkin lymphomas, AIDS(endemic)	40
Brain lymphoma, AIDS related	95
Brain lymphoma, immunocompetent hosts	5
Post-transplant lymphoproliferative disorder	95
Burkitt lymphoma, African (endemic)	>95
Burkitt lymphoma, North American	20
Burkitt lymphoma, AIDS-related	30
Lymphoma, primary immunodeficiency	Most
Pyothorax-associated lymphoma	90
Lymphomatoid granulomatosis(B cell lymphoma)	90
Plasmablastic lymphoma, AIDS-related	60
Primary effusion lymphoma, AIDS related	70
Age-related EBV-associated B cell lymphoproliferation	100*
Angioimmunoblastic T cell lymphoma (EBV+B cells)	80
Peripheral T cell lymphoma, unspecified	40
Extranodal NK/T lymphoma, nasal type	>95
NK leukemia	Most
γ δTcell lymphoma, mucosal	Most
T cell lymphoma in chronic active EBV infection	Most
*By definition the disease is EBV-related.	

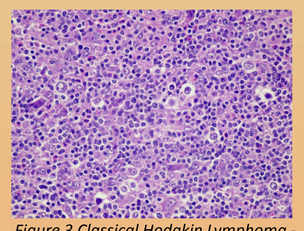


Figure 3 Classical Hodgkin Lymphoma - Nodular sclerosis - H & E - 40X

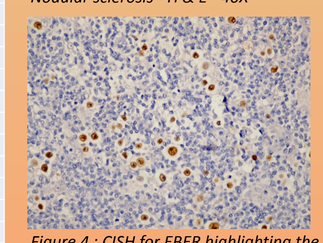


Figure 4 : CISH for EBER highlighting the Reed Sternberg cells in HL-NS – 40 X

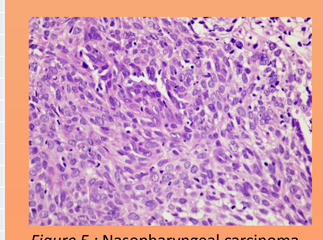


Figure 5 : Nasopharyngeal carcinoma - H & E – 40 X

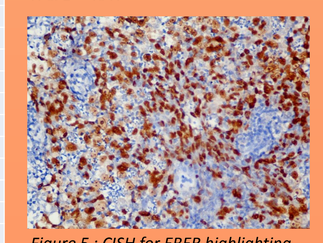


Figure 5 : CISH for EBER highlighting the tumour cells – 40 X

The positive cases in our modest pilot study were either of Classical Hodgkin Lymphoma or Nasopharyngeal carcinoma.
As early as 1966 MacMahon [7] proposed that Hodgkin's disease might be caused by an infectious agent. The first evidence that this agent might be EBV was provided by the detection of raised antibody titres to EBV antigens in patients with Hodgkin's disease.[8]
With the advent of cloned viral probes and Southern blot hybridization methods, EBV DNA was initially detected in 20–25% of Hodgkin's disease tumour specimens.[9]
However, this approach could not determine the locality of the EBV genome in tissues. In situ hybridization methods to detect EBV DNA provided the first demonstration of its existence in the HRS cells.[10]
Subsequently, the demonstration of the abundant 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.[10]
In Hodgkin's disease, the bulk of viral genomes are found in monoclonal form, indicating that infection of the tumour cells has occurred before clonal expansion.[11]
EBV appears to persist throughout the course of Hodgkin's disease and is also found in multiple sites of Hodgkin's disease.[12] In our study also we were able to demonstrate EBER in nodal & extranodal locations.
EBV has been detected in the malignant Reed-Sternberg/Hodgkin (RS/H) cells in approximately 40% of patients with Hodgkin disease.[1]
Of the 4 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[13]. We noticed a similar incidence with ***63.6%%*** mixed cellularity cases positive for EBER.
Various studies have shown that EBV positive rates are higher in male patients than in female patients.[14] We noticed a slight male preponderance as well.

Guidelines for Interpreting Epstein – Barr Virus –Encoded RNA (EBER) In situ Hybridization[1]

- *The morphologist must be competent in distinguishing Read sternberg /Hodgkin (RS/H) cells from nontumor cells. The cytologic features and distribution of RS/H cells should be assessed on matched H & 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 1 unequivocal RS/H cell a cases positive, Equivocal cases frequently are resolved when EBER and H & E stains are evaluated in parallel*
- *The EBER signal 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.*

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 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 IgG and IgA antibody titers to VCA 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.[15]
Additionally, 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.
Though EBV is certainly implicated in the pathogenesis of NPC, the exact mechanism and pathway by which it exerts its effects are unknown. Nevertheless, EBV studies, particularly circulating plasma EBV DNA levels, have shown utility in staging, prognosis, and post-therapeutic monitoring. Additionally, 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. [17]

We were not able to demonstrate EBER in other published associations but as the number of other diagnoses ranges from 1 to 3, it would be premature to comment. We will be prospectively continuing with this study.

CONCLUSION

- ***EBV*** is one of the best tumor markers yet discovered.
- EBV viral load testing has been incorporated into routine care of patients with PTLD, nasopharyngeal carcinoma, and AIDS lymphoma of the brain
- ***EBER in situ hybridization*** 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.

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