

Determination of the Expression of Latent Epstein Barr Virus in Omani Nasopharyngeal Carcinoma Patients

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Nasopharyngeal carcinoma(NPC) is a rare malignant carcinoma that develops in the epithelial lining of the nasopharyngeal mucosa. It is the most common neoplasm of the Nasopharynx and it is associated with many risk factors; one of them is Epstein-Barr virus infection. An Epstein-Barr virus is a tumorigenic herpes virus that infects and persists in B-lymphocytes without causing disease. This virus is associated with significant pathological conditions, such as benign and malignant lymphoproliferation. The aim of the present study is to determine Epstein -Barr encoded RNA 1&2 (EBER1,2) and latent membrane protein (LMP) expression in formalin-fixed paraffin-embedded tissue samples obtained from Omani patients diagnosed with nasopharyngeal carcinoma. Also, to identify the pattern and the type(s) of cells infected with EBV in nasopharyngeal carcinoma tissue samples obtained from Omani patients. Moreover, to compare the sensitivity of Immunohistochemistry and *in-situ* Hybridization for the detection of EBV in the nasopharyngeal carcinoma tissue sample. Thirteen formalin-fixed paraffin-embedded nasopharyngeal carcinoma tissue samples archived from the period 2010 to 2017, were obtained from the Pathology Departments of Sultan Qaboos University Hospital and the Armed Force Hospital. These tissue samples were processed using two different methods Immunohistochemistry (IHC) and *In situ* hybridization (ISH). Eleven out of thirteen NPC Omani patients were positive for EBV (84.61%) by either LMP-IHC or EBER-ISH. All cells stained positive for EBV in NPC tissue samples was of malignant type rather than normal cell type. EBV is mostly detected in patients in the age group of less than 50 years old. Also out of the 13 NPC patients, seven females (58.34%), and six males (46.15%) were positive for EBV. This study may provide evidence indicating an association between EBV and nasopharyngeal carcinoma. In addition, the detection of EBV in NPC obtained from Omani patients may encourage the physician to consider using anti-herpes virus drugs in the treatment of EBV positive NPC patients as an additional tool for the treatment of this kind of malignancy.

Keywords: Nasopharyngeal Carcinoma, Epstein Barr virus, Omani patients.

Nasopharyngeal carcinoma is a rare malignant carcinoma that develops in the epithelial lining of the nasopharyngeal mucosa¹. It is the most common neoplasm of the nasopharynx, and

it differs from other cancers of the head and neck in its occurrence, unique etiological associations, clinical behaviour and treatment². The incidence of this type of malignancy is very rare (less than 1

in 100.000) in most populations³, but it is relatively common in males of Western North Africa⁴. In Asian countries, 50 years old is generally the mean age of NPC patients⁵. In the Omani population, 40% of the total cases of NPC were reported from the capital city Muscat, and about 21% were reported from Ash Sharqiyah region⁶. Besides, 17% of cases were reported from both Al-Batinah and Dhofar regions⁶.

In high-risk areas, the undifferentiated carcinoma of nasopharyngeal type (UCNT) is the most dominant histopathological type³. Moreover, NPC associated risk factors include genetic susceptibility, Epstein Barr virus (EBV) infection, ecological risk factors^{3,7}. Also, certain dietary factors such as salted fish consumption is another factor that may contribute to the development of NPC⁶. Elevated antibody titers against Epstein Barr virus and a certain human leukocyte antigen class I genotypes are considered as well as a risk factors of NPC⁸.

Epstein-Barr virus (EBV) was first identified in a biopsy culture obtained from Burkitt lymphoma patients in 1964⁹. EBV is a tumorigenic herpesvirus that infects and persists in B-lymphocytes without causing disease⁹. In most cases, infection with EBV remains latent; however, in a few cases, it is associated with significant pathological conditions, such as benign and malignant lymphoproliferation⁹.

This virus has shown the ability to contribute to oncogenesis as it frequently detected in tumour tissue samples like Burkitt lymphoma, Hodgkin's disease, and post-transplant B cell lymphomas¹⁰. In UCNT (The undifferentiated carcinoma of nasopharyngeal type), the EBV infection presents at an early stage of tumour development, which is evident by the usual presence of the EBV genome in a circular and monoclonal episomal form in the tumour cells¹¹.

Latent membrane protein 1 (LMP1) which is a critical EBV oncogene³, is a transmembrane protein with two important properties, the first is its potent cell signalling properties and the second is the tumorigenic transformation properties¹². LMP1 is capable of inducing cancer progenitor cell-like phenotyping in epithelial cells, which may contribute to the progression of nasopharyngeal carcinoma¹³. Transformation and immortalization of cells by LMP1 is done through

directing the signalling pathways that stimulate cell proliferation and block apoptosis¹⁴. Many different downstream pathological changes can be caused by LMP1 including cell proliferation, apoptosis and metastasis¹⁵. LMP has the capabilities of activating several oncogenic signalling pathways such as NF- κ B, JNK and MAPK¹⁵.

Epstein-Barr virus-encoded RNA (EBER), which have two types EBER1 and EBER2, is a non-polyadenylated untranslated RNAs of 167 (EBER1) and 172 (EBER2) nucleotides long, and it is transcribed by RNA polymerase III¹⁶. Besides, both EBER1 and EBER2 contribute to the oncogenesis of EBV, the transforming ability of recombinant EBVs expressing EBER2 was as high as that of EBVs expressing both EBER1 and EBER2¹⁷. The way by which EBERs contribute to the efficient growth transformation of B-lymphocytes is by enhancing the growth potential of the transformed lymphocytes¹⁶.

MATERIALS AND METHODS

Specimens

Thirteen formalin-fixed paraffin-embedded nasopharyngeal carcinoma tissue samples collected and archived from the period 2010 until 2017, were included in the present study. These samples were obtained from the Pathology Departments of SQUH and the Armed Force Hospital in Muscat/Oman. Also, the patient information including diagnosis, age, gender and MRN of each sample was recorded. The research ethical approval for the present study was obtained from the Research Ethics Committee of the College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman (MREC # 1495).

Tissue processing

Tissue processing for both Immunohistochemistry (IHC) and *in situ* hybridization (ISH), were carried out as described previously^{18,19}. Briefly, the nasopharyngeal carcinoma formalin-fixed paraffin-embedded tissue samples were cut into sequential 4 μ m sections using the microtome (Thermo Scientific).

From each tissue sample, 3 sections were cut and one was used for Haematoxylin and Eosin staining (H&E) and the other 2 sequential sections bounded covalently to the glass slides, where one was used for *in situ* hybridization (ISH) and the

other one was used for Immunohistochemistry (IHC) analysis. After sectioning the slides were incubated in 60 °C oven for one hour.

Positive controls consisted of Hodgkins Lymphoma tissue sample was included in each experiment. In addition, a negative control, was included in each experiment in which the primary antibody /EBERs probe was omitted and replaced with a buffered saline.

Immunohistochemistry

Immunohistochemistry was carried out on nasopharyngeal carcinoma tissue(NPC), samples using the Envision Flex+ High pH (Ref K8002, Dako) in accordance with the manufacturer's instructions. Prior to staining the sections were deparaffinized twice through two changes of xylene, each xylene change was carried out for 5 minutes. Then, sections were hydrated in three changes of graded ethanol, each for 5 minutes followed by heat-induced epitope retrieval using the target high pH solution in the detection kit according to the package insert for the primary antibody. Subsequently, tissue sections were incubated in EnVision Flex Peroxidase-Blocking Reagent (SM801) for 10 minutes to block the endogenous peroxidase activity. This was followed by washing in Envision Flex Wash Buffer (TBS-DM831) (Dako, Denmark) for 5 minutes. Immunohistochemistry on processed tissue sections was carried out by the incubation of NPC tissue sections for 60 minutes at 37°C with primary antibody consisted of mouse monoclonal antibody against EBV-encoded LMP (clone: CS, 1-4 and isotype: IgG1, kappa, Dako, Denmark), followed by incubation at room temperature for

30 minutes with biotinylated secondary antibody. The secondary polyclonal antibody used in the present study consisted of dextran coupled with peroxidase molecules (Dako Envision Flex/HRP SM802). Then, 3,3-diaminobenzidine tetra hydrochloride (DAB) was add as a chromogen. The DAB chromogen gave a brown colour stain over the target site (EBV LMP protein). Mayer's Haematoxylin stain was used as a counterstain.

In situ hybridization (ISH)

The expression of EBER1 and EBER2 of EBV in NPC tissue samples was carried out by using the Inform EBER Probe (800-2842, Ventana Medical Systems, Roche Diagnostics GmbH, Mannheim Germany). The EBERs positive signals in tissue samples were detected by Ventana ISH/View Blue detection Kit (Ref: 853-2193). *In situ* hybridization experiment was performed using (Ventana Bench Mark Ultra, Medical Systems Inc, Tucson, AZ, USA) following manufacturer instructions.

Data analysis

SPSS version 23 was used for data analysis. Frequency tables and pie charts or bar charts have been used to display percentages for categorized variables, and continuous variables have been shown as Mean \pm SD and error bar charts. For the analysis, to test the significance of the association between categorized variables, the chi-square test was used.

RESULTS

Epstein-Barr virus (EBV) expression in NPC tissue samples

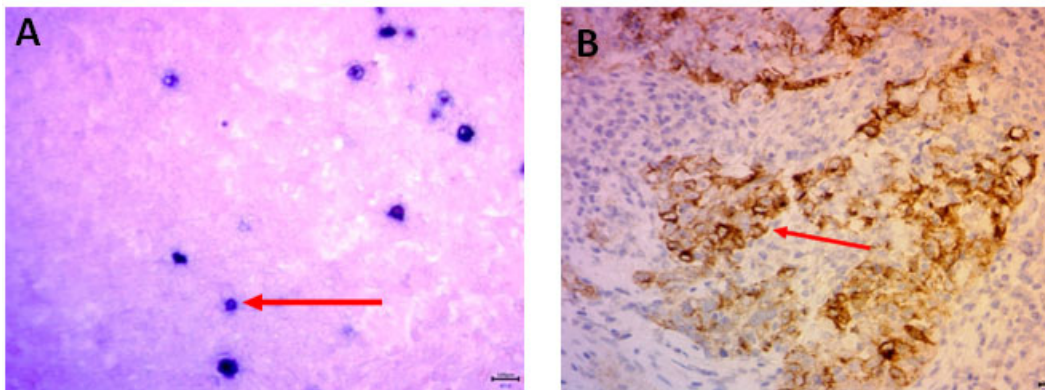


Fig. 1. Nasopharyngeal carcinoma tissue samples. (40x),

A: ISH demonstrating, positive signals for EBER-EBV appear as purple color stain (red arrow- head). B: IHC demonstrating positive signals for EBV-LMP appearing as brown color stain (red arrowhead).

The expression of Epstein-Barr virus (EBV) was detected in the present study by using two different technical approaches, *In-situ* hybridization for the detection of EBER and Immunohistochemistry for the detection of LMP protein of EBV in formalin-fixed paraffin-embedded NPC tissue samples obtained from Omani patients.

The results for the expression of EBV-LMP and the expression of EBV-EBER are summarized in Table 1. Positive cases were considered positive if they are positive by either one of the techniques (ISH or IHC). Eleven out of thirteen NPC Omani patients' tissue samples were positive for EBV (84.61%) by either LMP-IHC or EBER-ISH; five of them were positive by both LMP-IHC and EBER-ISH (about 38.46%) while

four of them were positive only by EBER-ISH (about 30.76%). Also, one of the cases was positive by LMP-IHC only. The results of EBER-ISH and LMP-IHC NPC positive cases are shown in Table 1 and Figure 1. The EBV positive NPC tissue samples detected by EBER-ISH (69.23%) is higher than that of LMP-IHC (53.84%). This may indicate that the sensitivity and specificity of EBER-ISH are higher than that of LMP-IHC for the detection of EBV.

The staining pattern of EBV expression/lymphocytic infiltration

The pathological examination of the NPC tissue sections stained with Haematoxylin and Eosin and the interpretation of IHC and ISH results was carried out by independent Pathologist. In the seven positive cases, latent Membrane

Table 1. The expression of EBER and LMP in Nasopharyngeal carcinoma in Omani patients

EBER-EBV positivity by ISH (%)	LMP-EBV positivity by IHC (%)	Total positive cases	Total number of cases
9 (69.23%)	7 (53.84%)	11 (84.61%)	13 (100%)

Table 2. The integrity of staining, staining patterns and the type cells stained positive by LMP-EBV

Cases	Integrity of staining	Staining pattern	Type of cells stained positive
Case 1	Mild	Cytoplasmic	Malignant cells
Case 2	Moderate	and	
Case 3	Moderate	Membranous	
Case 4	Mild		
Case 5	Strong		
Case 6	Moderate (patchy)		
Case 7	Mild		

Table 3. The integrity of staining, staining patterns and the type cells stained positive by EBER-EBV

Histo#	Integrity of staining	Staining pattern	Type of cells stained positive
Case 1	Strong	Nuclear	Malignant cells
Case 2	Strong	Nuclear	Malignant cells
Case 3	Strong	Nuclear	Malignant cells
Case 4	Strong	Nuclear	Malignant cells

Protein (LMP) staining of EBV was Cytoplasmic and Membranous [Figure.1: B, Figure.2: C, E,G] [Table.2], while in EBV positive cases the staining pattern was Nuclear [Figure.1:A; Table.3]. The integrity of the staining in LMP-IHC ranges from mild to moderate to strong (Table 2; Figure.2: C, E, G); however, strong staining integrity was seen in EBV-ISH [Table.3; Figure.1:A]. In addition, all cells stained positive by both ISH and IHC was of malignant type rather than normal cell type.

Examples of staining pattern of EBV-ISH and LMP-IHC positive cases are shown in Figures.1,2.

The degree of lymphocytic infiltration in correlation to positive and negative cases was detected. Mild to moderate (7.143%), moderate (35.71%), and moderate to severe (7.143%) lymphocytic infiltration was observed in positive cases with LMP-IHC, while negative cases showed a similar degree of sparse, mild, and moderate of lymphocytic infiltration(16.67%) [Figure 2A]. Also, Haematoxylin and Eosin stain for NPC tissue

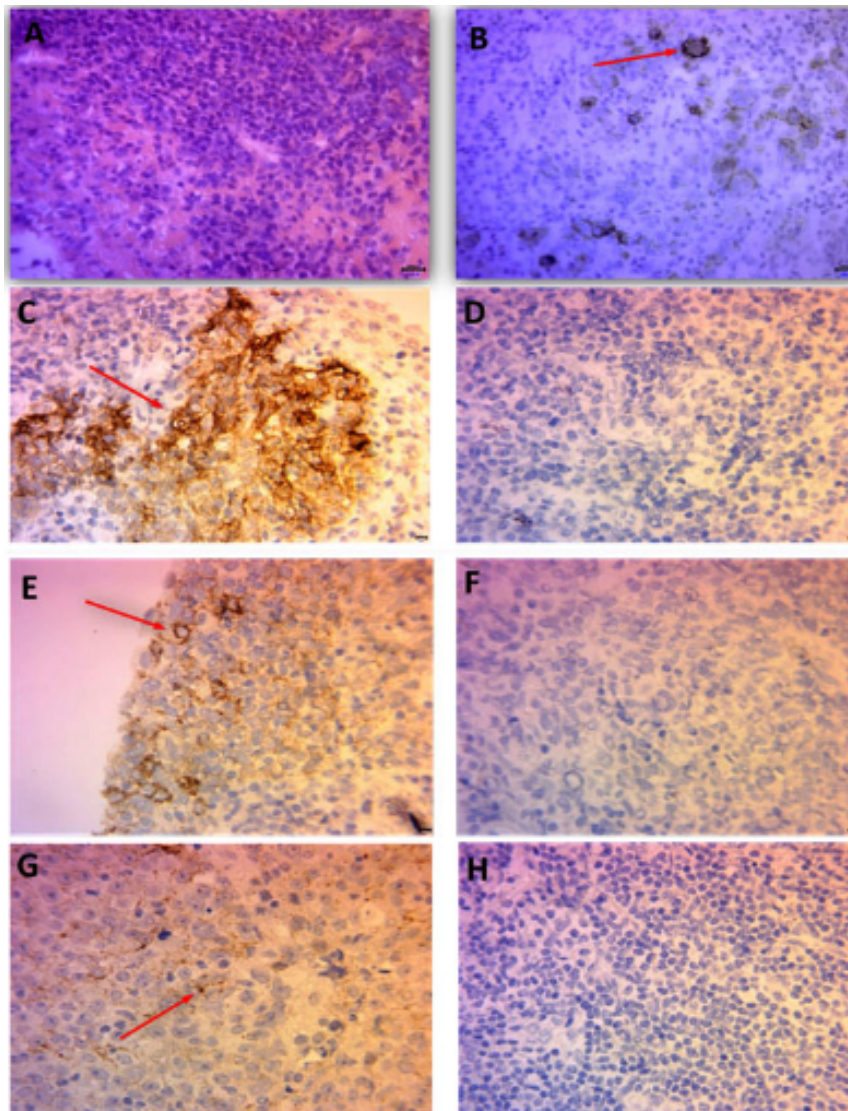


Fig. 2. Nasopharyngeal carcinoma tissue samples, A: lymphocytic infiltration (HE Stain) (40X), B: positive control (Hodgkin lymphoma) for LMP-IHC (40X), C: Strong positivity for LMP-IHC (60X), D: Negative control for C (60X), E: Moderate positivity for LMP-IHC (60X), F: Negative control for E (60X), G: Weak positivity for LMP-IHC (60X), H: Negative control for G (60X)

samples showed a fibro collagenous tissue densely infiltrated by lymphocytes [Figure.2: A].

EBV gene expression in correlation to age and gender

Thirteen NPC patients (from 2010-2017) were divided into two age groups, < 50 years old and ≥50 years old. The first group (< 50 years) includes seven cases, four of them were positive

for LMP-IHC (57.14%), while five of them were positive for EBER-ISH (71.42%). The second age group (≥50 years) included 6 cases, three of them were positive for LMP-IHC (50%), and four of them were positive for EBER-ISH (66.66%) [Table.4] [Figure.3]. The P-Value for both positivity by LMP-IHC and EBER-ISH shows a value higher than 0.05, which means that the

Table 4. The frequency of EBV positive cases in NPC (2010-2017) in correlation with age

Age	Number of Cases	LMP-EBV Positivity by IHC (%)	EBER-EBV Positivity by ISH (%)
< 50 years	7	4 (57.14%)	5 (71.42%)
≥ 50 years	6	3 (50%)	4 (66.66%)
Total	13	7	9
P-value		0.797	0.853

Table 5. The frequency of EBV positive cases in NPC (2010-2017) in correlation with Gender

Gender	Number of Cases	LMP-EBV Positivity by IHC (%)	EBER-EBV Positivity by ISH (%)
Female	7	5 (71.42%)	6 (85.71%)
Male	6	2 (33.33%)	3 (50%)
Total	13	7	9
P-value		0.17	0.164

The frequency of EBV positive cases in NPC (2010-2017) in correlation with Gender and Age

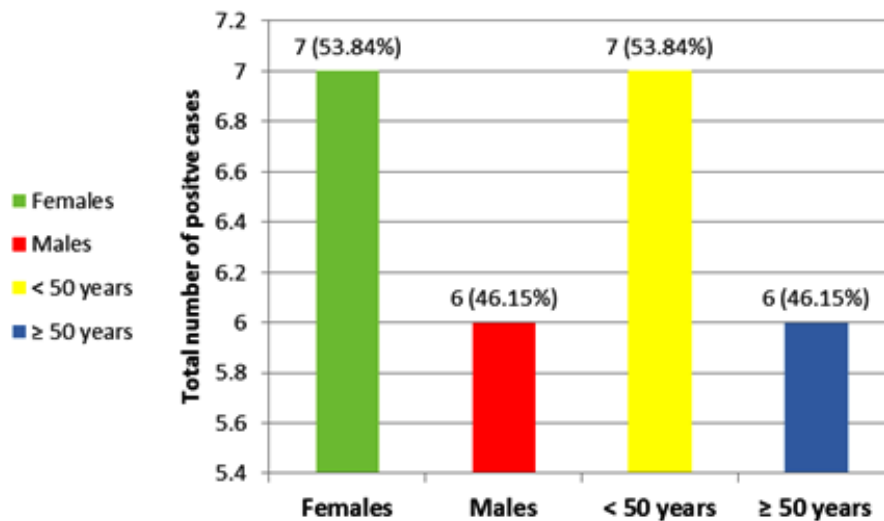


Fig. 3. The frequency of EBV positive cases in NPC (2010-2017) in correlation with Gender and Age

association between the age and the positivity of both LMP and EBV is not statistically significant.

In relation to gender, out of the thirteen cases included in this study seven of them were female, and the rest six were males. Five out of the seven females were positive for LMP-IHC (71.42%), while six of them were positive for EBV-ISH (85.71%). In the male's category, two of them were positive for LMP-IHC (33.33%), and three of them were positive for EBV-ISH (50%) [Table.5] [Figure.3]. The P-Value for both positivity by LMP-IHC and EBV-ISH shows a value higher than 0.05, which means that the association between the gender and the positivity of both LMP and EBV is not significant.

DISCUSSION

Nasopharyngeal carcinoma has a very rare incidence (less than 1 in 100,000) in most populations³. The mean age of developing NPC worldwide is of 50 years old in most Asian countries⁵. In contrast, this study shows that the mean age of Omani patients with NPC was 47 years old, which could be considered close to that of Asian countries. The number of samples in the present study (Thirteen cases) demonstrates the very rare incidence of this type of malignancy in Oman⁶.

There are different techniques used for the detection of Epstein-Barr virus (EBV), including immunohistochemistry (IHC), *in situ* hybridization (ISH), and polymerase chain reaction (PCR). The present study showed that eleven out of thirteen NPC Omani patients were positive for EBV by either LMP-IHC or EBV-ISH (84.6%), however, the positivity of EBV-ISH (69.23%) is higher than that of LMP-IHC (53.84%). This indicates that the sensitivity and specificity of EBV-ISH are higher than that of LMP-IHC for the detection of EBV. However, these findings are not of that significance as this could be due to the low number of samples used in the present study. This finding is similar to some degree to the finding of another study, which showed that EBV-ISH is more sensitive and more reliable than LMP-IHC for the detection of EBV in formalin-fixed paraffin-embedded tissue samples²⁰.

Most of the EBV positive cases were found in the female category (58.34%), precisely out of the thirteen cases included in the present study

seven of them were female, and the rest six were males (46.15%). This shows that EBV associated Nasopharyngeal carcinoma is more common in females than males among patients in Oman. This is different from the findings of other studies, which found that EBV is more prevalent in male NPC patients than females⁷. However, in the present study, the P-Value for both positivity by LMP-IHC and EBV-ISH in correlation to age and gender shows a value higher than 0.05, which indicates that the association between both the age and the gender and the positivity of both LMP and EBV is statistically non-significant. In addition, the results of the present study, show that EBV associated nasopharyngeal carcinoma are more prevalent in patients with the age of less than 50 years old (53.84%) in comparison to the age group ≥ 50 years old (46.15%). This is similar to a study carried out on the Sudanese population which found that the EBV associated NPC were seen mostly among the age group of 21-40 years old²⁷. Moreover, in the present study, all cells stained positive were of malignant type rather than normal cell type. This may indicate a relation between Epstein Barr Virus (EBV) and the pathogenesis of nasopharyngeal carcinoma.

The degree of lymphocytic infiltration ranged from sparse to mild to moderate to severe in some cases. In addition, the results of the present study showed that there was a correlation between the positivity for LMP-IHC and the degree of lymphocytic infiltration; where a higher degree of lymphocytic infiltration was detected in positive cases for LMP-IHC. Similarly, other studies validate this correlation between the positivity of EBV and the consistency of lymphocytic infiltration^{21,22}.

In conclusion, this study aimed to determine the expression of latent Epstein-Barr virus in Omani nasopharyngeal carcinoma patients. The importance of this study originated from the need to determine the prevalence of EBV in Omani patients with NPC especially that the prevalence of latent EBV infection is considered as a risk factor of NPC. The present study showed a clear association between EBV and NPC among Omani patients, especially, that all cells stained positive for EBV were of malignant type rather than normal cell type. This indicates that EBV infection may play a role in the pathogenesis of NPC. The detection of

EBV in NPC obtained from Omani patients may encourage the physician to consider using anti-herpes virus drugs in the treatment of EBV positive NPC patients as an additional tool for the treatment and the management of this kind of malignancy.

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Conflict of interest

The authors declare no conflict of interest

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