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Epstein-Barr virus RNA as a diagnostic biomarker in gastric adenocarcinoma

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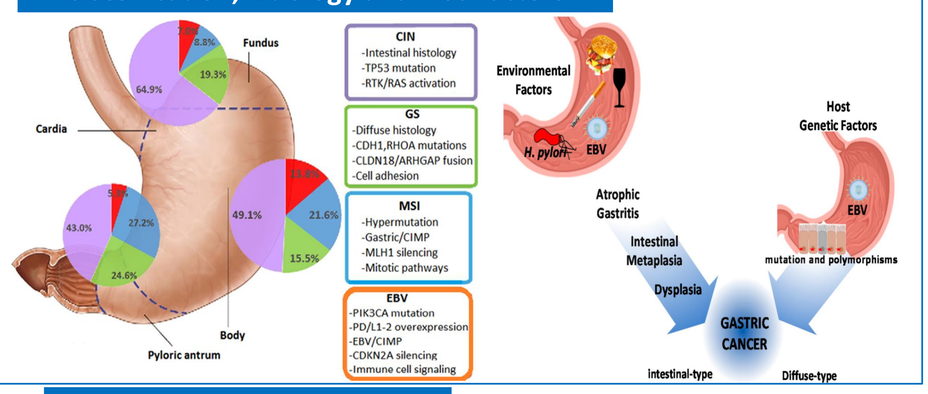
BACKGROUND

Gastric cancer (GC) remains the fourth cause of cancer-related death worldwide. Epstein–Barr virus-associated gastric cancer (EBVaGC) is a distinct molecular subtype, it accounts for up to 10% of all molecular subtypes of gastric cancer. After entering the incubation period in human bodies, cells infected with EBV transcribe small non-coding RNAs called EBER. The EBV prompts methylation of the host genome, imbalance of the cellular signaling pathway, abnormal gene expression, generation of a tumor microenvironment of infected gastric epithelial cells, and initiation and development of GC. Moreover, EBERs are involved in reduced E-cadherin expression, which is a key step in the carcinogenesis of EBVaGC. In addition many studies find EBV infection to be associated with p53 methylation and Deregulation of the PI3K/Akt/mTOR pathway . The immune response and tumor microenvironment play an essential role in the progression of EBVaGC. With recent developments in immunotherapy, better treatment of EBVaGC patients appears achievable.

OBJECTIVES

Our study investigated the involvement of EBV in GC and the specificities of the associated tumor microenvironment in Algerian population.

Classification, Etiology and Risc Factors



ASSAY WORKFLOW

We reported the cases of two patients selected from 20 gastrectomy specimens, based on the presence of peri-tumoral inflammatory infiltrate. Epstein–Barr encoding region (EBER) in situ hybridization (CISH) is the methodology of choice for the detection of the Epstein–Barr virus (EBV) in tissue sections. The evaluation of inflammatory infiltrate markers; CD3, CD4, CD8, CD79, MPO and CD68 as well as the expression of the oncogenic markers; p53, E-cadherin, β-catenin and mTOR have been assessed by Immunohistochemistry (IHC).

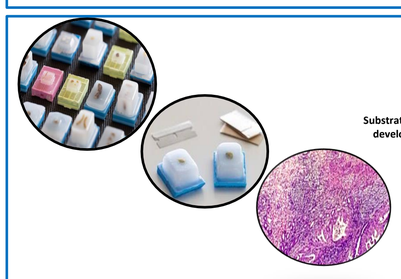


Figure 1: A study population. Selection was based on the presence of a Peritumoral inflammatory infiltrate

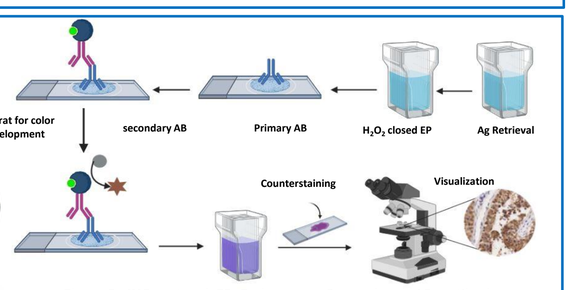


Figure 2 . IHC protocol EP: Endegenous Peroxydase , AB: antibody

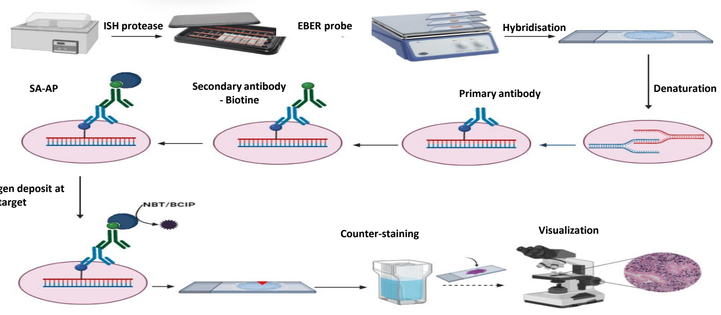


Figure 3. Chromogenic in situ Hybridisation (CISH) SA-AP Streptavidine – Alkaline Phosphatase . The manual adaptation of the automated CISH protocol on EBER-negative and Positive Specimen Slides has been validated by the Molecular Biology Unit of the Pathological Anatomy Department (CHU Douera).

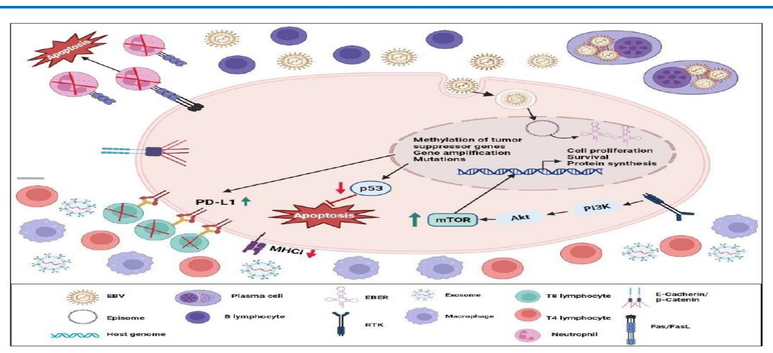


Figure 8 : Summary of EBV-mediated gastric carcinogenesis

RESULTS

This study examined two GC male patients, aged 58 years on average, who presented after undergoing chemotherapy and Gastrectomy. The patients had different tumor sizes and locations and were in the T3N1 and T3N2 stages respectively (Table I). Histological examination revealed a moderately differentiated adenocarcinoma and a moderately differentiated papillary-type adenocarcinoma with lymphoid follicles, respectively, in the two patients (Fig.4). Chromogenic in situ hybridization (CISH) was EBV+ in the second patient (Fig.5). The immunohistochemistry (IHC) indicated a Crohn-like feature related to EBVaGC, characterised by a strong recruitment of CD79+ B lymphocytes and CD68+ macrophages (Figs.6A and C). The low CD8+/CD4+ ratio (<1) suggested a defect of endogenic antigenic presentation in EBV+ cells due to the EBV infection or PD-L1 overexpression in EBV+ cells which inhibits TL proliferation (Fig.6B). The low expression of MPO was associated to EBV infection as immune escape strategy (Fig.6C), probably due to Neutrophiles suppression via Fas signalling pathway. The membrane β-catenin and E-cadherin were highly expressed (Fig .7A). The E-cadherin/β-catenin complex is very important in maintaining epithelial morphology, and can be disrupted during oncogenesis. The parallel low expression of P53 (4.5% *vs.* 80%) and the over-expression of mTOR (71% *vs.* 15%) in EBV⁺ patient, confirmed a loss of anti-tumor function and the alteration of the PI3K/Akt/mTOR pathway (Figs. 7B and C).

1. Clinicopathological characteristics

Table I: General data of the clinicopathological characteristics

Patients	Age	Sex	Sample	Tumour size	Topography	TNM state	Chemotherapy
1	50	M	Total Gastrectomy	5x7x3cm	Cardia	T3N1	Post-chemotherapy
2	67	M	Total Gastrectomy	7x4x1.5cm	Antrum	T3N2	Post-chemotherapy

2. Histological appearance of gastric tumors (H&E)

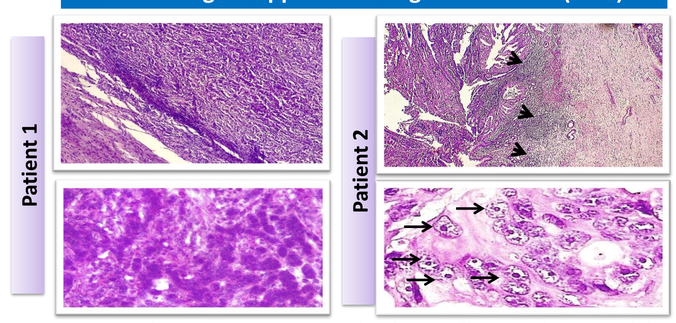


Figure 4. Histological appearance of gastric tumors. **P1:** Moderately differentiated adenocarcinoma with significant inflammation (HEx100, High magnification x400); **P2:** Moderately differentiated papillary adenocarcinoma presenting a dense inflammatory infiltrate and a few lymphoid follicles (**arrow head**) (HE x100), Eosinophilic intranuclear inclusion (**arrow**)(High magnification x400).

3. EBER detection by Chromogenic in situ Hybridisation (CISH)

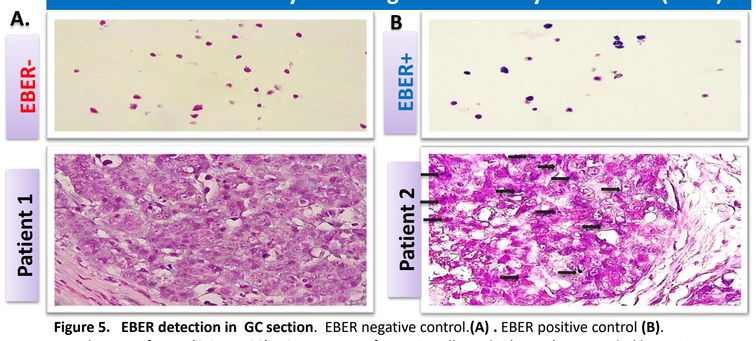


Figure 5. EBER detection in GC section. EBER negative control.**(A) .** EBER positive control **(B)**. **P1:** Absence of EBER (CISH, x400). **P2:** Presence of EBER in cells nuclei (**arrow**) surrounded by an important inflammatory infiltrate (CISH, x400). The RBER⁺ case is characterized by more than three lymphoid follicles with active germinal centers, suggesting a Crohn-like subtype of EBVaGC. This is classified as intestinal-type adenocarcinoma, the most common type, according to Lauren's classification.

4. Immunohistochemical characterisation of the immune infiltrate in tumour microenvironment

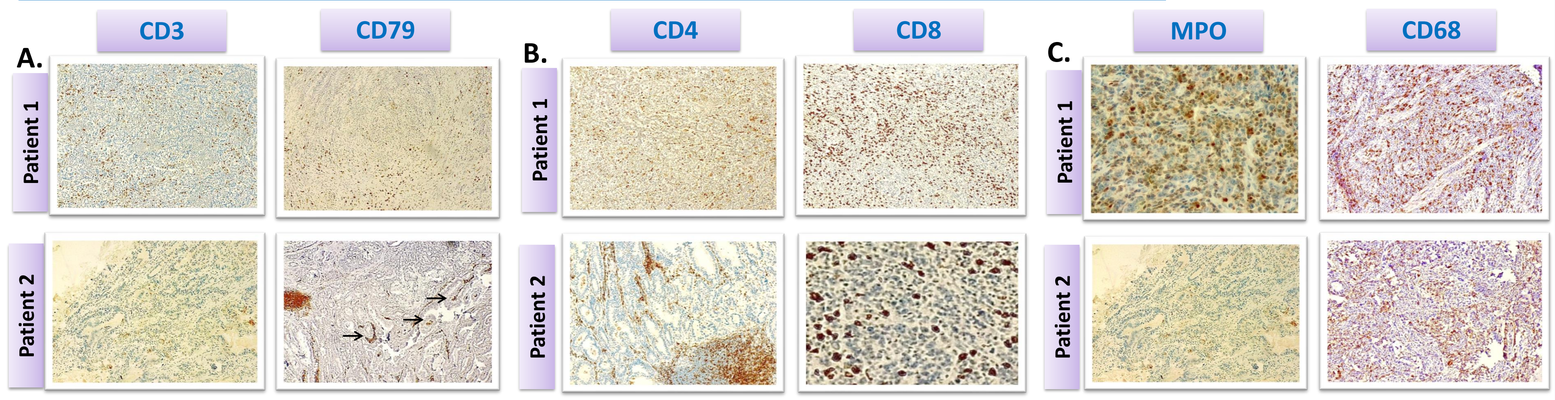


Figure 6. T and B Lymphocytes and leucocytes subsets characterisation. IHC analysis indicated that both patients had a polymorphic inflammatory infiltrate, which included CD79+ LB, CD4+ , CD8+ LT, CD68+ macrophages, and MPO+ neutrophils. The examination of lymphocyte populations revealed that both patients had a significant level of LT CD3+ and LB 79+ . In patient 2, the 79+ LBs were found in lymphoid follicles (**arrow**), while in patient 1, they were present as isolated cells **(A). Patient 2** Showed an LT CD8 + / LT CD4 + Ratio <1 comparer to **Patient 1** who presented an LT CD8 + / LT CD4 + Ratio =1 **(B) .**The levels of CD68+ macrophage were equal in both patients . MPO analysis revealed the presence of polynuclear neutrophils in **patient 1** and a weak presence in **patient 2 (C)**. **(A)** CD3 and CD79 (IHC, x100). **(B)** P1: CD4 and CD8 (IHC, x100). **P2**: CD4 (IHC, x100), CD8 (IHC, x400). **(C). P1**: High expression of MPO (IHC, x400), **P2**: low expression of MPO (IHC,x100), same level of CD68 (IHC x400)

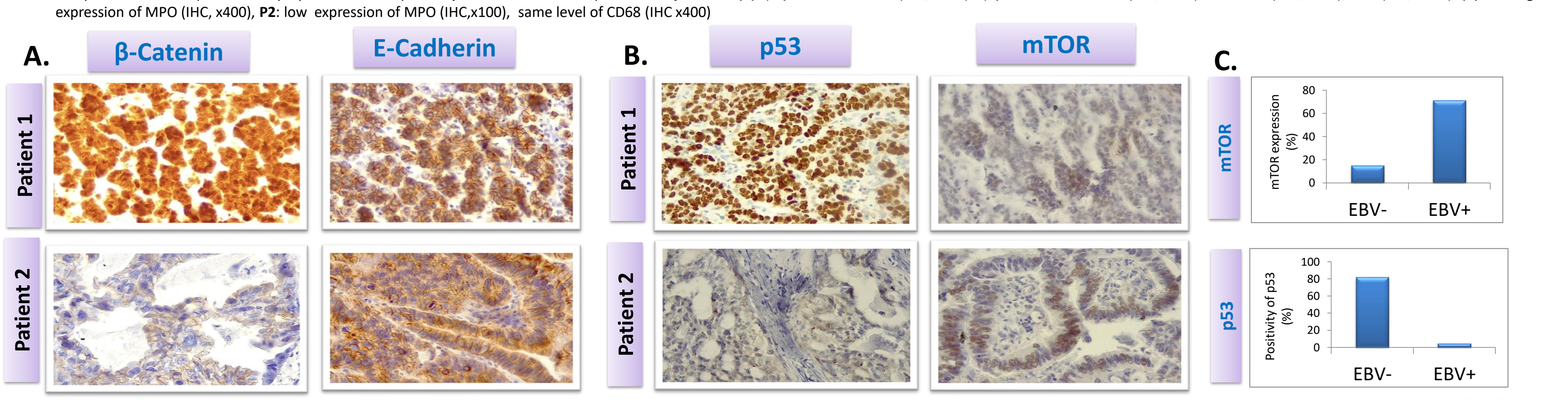


Figure 7. Expression of β-catenin and E-cadherin, P53 and mTOR. Assessment of **β-catenin** expression by IHC revealed **high nuclear expression** in patient 1, in contrast to patient 2 who had **membrane expression (A).** The **E-cadherin** is membranar in the two patients **(A).** The two patient had expressed different levels of **P53** and **mTOR (B).** The expression of **P 53** was <5 % in patient 2 , while patient 1 showed **an overexpression of P53** (80%) **. Overexpression of nucleocytoplasmic mTOR** in patient 2 at 71% **(C) and low** nuclear expression in patient 1 (15%) **(C).**
P1: nuclear expression of β-catenin (IHC, x400). **P2:** membrane expression of β-catenin (IHC, x400), membrane expression of E-cadherin (IHC, x400) . **P1:** Low nuclear expression (IHC, x400). **P2**: nucleocytoplasmic overexpression (IHC, x400). **P1**: nucleocytoplasmic overexpression p53 (IHC, x400). **P2**:Low expression of p53 (IHC, x400).

CONCLUSION

EBVaGC seems to have a very good prognosis , however, the underlying mechanisms of the cellular immune response have not been thoroughly elucidated. Therapeutic approaches that are currently being studied for the treatment of GC include anti-PI3K inhibitors, monoclonal antibodies, Akt inhibitors, anti-PD-L1 monoclonal antibodies, and demethylating agents (Fig.8). Histological hallmark such as marked peri-tumoral immune cell infiltration in tumors microenvironment and positivity of EBER in CISH are linked to a favorable prognosis and will be helpful in EBVaGC diagnosis. Our study aims to increase pathologists' awareness regarding the immune response of the host against tumors. This will help identify cases eligible for molecular diagnosis through in situ hybridization to detect EBV. It will aid in proposing clinical trials for such patients, helping search for therapies to improve their survival chances. Therefore, further researches are necessary to better understand the role of tumor microenvironment in EBVaGC.