Protein profile and search for diagnostic markers in gastric cancer patients in Algeria

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ABSTRACT

Gastric cancer is one of the most common cancers in the world with one million new cases each year. It remains the second leading cause of death by cancer for both sexes. In Algeria, it represents 5% of all cancers listed and it comes in the fifth position with 1430 new cases each year. Gastric cancer was associated with Epstein-Barr virus (EBV) with an incidence of 10 to 18%. The LMP-1 protein is considered as the major EBV oncogene. Thanks to its signaling, LMP1 presents immortalization and transformation properties, able of inhibiting cell differentiation. The most promising approach to reduce mortality from gastric cancer is the early diagnosis by identifying specific and sensitive biomarkers. It is in this context that we first realized electrophoresis on cellulose acetate plates of the sera of patients with gastric cancer. The proteinogram obtained are, in general, very similar and they have a uniform appearance like paths known to be normal. Some of them have hypo-proteinemia and hypo-albuminemia in relation with inflammation and protein losses by digestive bleeding. In a second step, we performed SDS-PAGES to compare protein profiles of sera of gastric cancer subjects with those of healthy subjects. We have noted the existence of an additional band of 27 kDa in 20% of gastric cancer topics we found a disparity concerning the 18 kDa band which is present in 65% of patients and only in 43% of subjects healthy. Then, we performed ELISA on sera and on protein extracts of gastric biopsies to look for the presence of the LMP1 protein. The latter was detected in about 50% of cases. In healthy subjects, the samples were negative. By statistical tests, we have shown that the protein is expressed without regard to the patient's age or stage of cancer progression. LMP-1 may thus, serve as a biomarker for the diagnosis of EBV positive gastric cancer.

Keywords: Gastric cancer, EBV-LMP1, SDS-PAGE, proteinogram.
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INTRODUCTION

Gastric cancer represents 7.8% of all new cancer cases, making it currently the fourth cancer in the world [1] [2]. It is the second leading cause of death with cancer in both sexes (736 000 deaths, 9.7%). His prognosis remains delicate with a survival rate of about 15% at 5 years [3].

Food or environmental risk factors such as alcohol and smoking, most likely, play a major role in the occurrence of the disease, in addition to this, the genetic factors. It has been demonstrated that the molecular mechanisms underlying gastric cancer include, most commonly, alterations in oncogene, tumor suppressor genes, cell cycle regulators, cell adhesion molecules and repair genes DNA [4].

Epstein Baar (EBV) is a ubiquitous virus with more than 95% of the adult world population that carries the trace of the primary infection. The oncogenic role of EBV was suspected very early, due to the ability of the virus to

immortalize human B cells *in vitro*. These cells contain the viral genome in the latent state, expressing different viral proteins [5].

Gastric cancers were associated with EBV infection since 1990. The association was first described in a rare form of gastric tumor, identical to EBV-positive carcinomas observed in other organs, including the nasopharynx, and known under the name of the undifferentiated carcinoma with lymphoid stroma [6]. It was later shown that all conventional gastric adenocarcinoma histological types can be linked to EBV infection [7].

LMP1 (Latent Membrane Protein 1) considered as major EBV oncogene and is responsible for many phenotypic changes in cells infected with EBV [8]. According to DAWSON [9], the expression of LMP1 led to the diversion and the chronic deregulation of cellular signaling pathways and their target genes involved particularly in proliferative processes.

Because gastric cancer presents discrete clinical symptoms, often late and nonspecific, indicate a disease already locally advanced or metastatic [10]. The identification of the disease in an early stage may be the most promising approach to reduce gastric cancer mortality, hence the need to search for sensitive and reliable biomarkers for Earlier Diagnosis.

MATERIALS AND METHODS

We collected 20 sera samples of gastric cancer patients at the University Hospital of Tizi-Ouzou, and 20 sera samples from healthy subjects and 15 biopsies of gastric cancer.

2-1-Serum protein electrophoresis on cellulose acetate
The analysis was conducted on 20 serum samples from subjects with gastric cancer. We assayed the total protein by the automatic method (Beckman Coulter CX9) adopting the principle of the Biuret method.

Cellulose acetate plate, are previously immersed in a buffer solution for 20 minutes. 10μl of serum is then deposited on the plate. Immediately after electrophoresis migration carried out at pH 8.6 and at a voltage of 180 volts for 16 minutes, the plate is disclosed in a coloring bath at ponceau red at 0.5% where it is completely immersed for 6 minutes. The plate was then distained in three successive baths of acetic acid at 5%, 2 minutes each.

The plate is transferred in a pure methanol bath where it is left dipping for 4 minutes. The plate is then transferred to a lightening solution composed of glacial acetic acid at 29%, of absolute methanol at 67% and of 4% lightening reactant for 10 minutes. In order to complete the brightening, the plate is dried in an oven heated at 37-40 °C for ten minutes.

At the end of the practical realization, the result is visible on the cellulose acetate plate and can then be read by a densitometer (Helena junior 24) using a wavelength of 525 nm.

2-2-SDS-PAGE of serum proteins in the presence of β-mercaptoethanol
We used the method of LAEMMLI [11] which involves using a biphasic system with a stacking gel of 4%, in 0.5 M Tris-HCl buffer pH 6.8 and a separating gel in Tris-HCl buffer 1.5M pH 8.8. In order to better separate proteins with the MM 50 to 10 kDa, we conducted our analyses on a separation gel 17%. The polymerization thereof is initiated by ammonium persulfate and TEMED.

The samples are first diluted 1/2 with a solution RIPA (Radio Immuno Precipitation Assay). Thus, depending on the serum proteins of each sample, and to make deposits of 70 to 100 micrograms of proteins per well, we pipetted the required amount of serum, supplemented with 5μl of denaturing solution to 4X, containing SDS, the β-mercaptoethanol and bromophenol blue.

The samples are placed in alternating wells between the sera of healthy subjects and subjects with gastric cancer. A size marker Page RulerTM More Prestained Ladder (Fermentas LIFE SCIENCE) is used. This kit includes 9 pre-stained proteins with the following MM: 250, 130, 95, 72, 55, 36, 28, 17 and 10 kDa. The migration of the proteins is performed at room temperature under a DC voltage of 200 V and a current of 20 mA for two hours and a half.

At the end of electrophoresis, the gels were removed, the proteins are first fixed in 12% TCA solution (w/v) for 45 minutes, and then stained for one hour by Coomassie Blue R250 in an amount of 0, 2% (w/v). To reveal the electrophoresis bands, we proceed to the discoloration of the immersion of the latter by gel in a mixture of water, methanol and acetic acid in proportions of 3.12 / 1.5 / 0.37 (v/v/v).

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2-3-Extraction of tissue proteins in gastric tumors
In order to achieve ELISA on gastric cancer biopsies, we proceed to the extraction of tissue proteins. For this purpose, it carried out a tissue grinding for each gastric tumor, then, we take back the cell pellets in 700µl of RIPA added with 70µl of anti-proteases (Trypsin Inhibitor type I-S from Soybean). The sample is then sonicated (Sonicator Advantage-Lab AL) twice for one minute at a power of 20 W amplitude in ice. It is finally centrifuged (centrifuge: Sigma 4-16 K) at 10,000 g at 4 °C for 15 min. The supernatant containing the protein is recovered.

2-4-Search of membrane protein LMP1 latency by ELISA
We estimated the value of the LMP-1 by using the ELISA test, which is best suitable for a clinical diagnosis. This search is performed on all serum samples and protein extracts obtained from gastric biopsies, and also on samples of healthy subjects sera.

3ul of serum or protein extract supplemented with 50ul PBS are incubated overnight at 4°C (coating). After washing, we proceed to the saturation of the plate with 2% BSA (in PBS) and incubated for one hour at 37 °C. 100 µl of monoclonal antibody LMP1 S12 are added to each well at 0.1 µg / ml and incubated at 37 °C for two hours. We add then, the anti-mouse peroxidase-coupled at 0.1 µg / ml antibody and incubated for 30 minutes at 37 °C. 50 µl/ml of tetramethylbenzidine (TMB) are later added and the plate is placed in the dark for 10 minutes at room temperature. To stop the reaction, 50 µl/ml phosphoric acid 0.1M and the reading is done at 450 nm by an ELISA reader (BioTek).

In this analysis, we used as a negative control serum derived from umbilical cord, which is supposed to contain no protein of the Epstein Barr virus, since not contaminated by it. We also used as a positive control protein extract cells from a lineage P3HR1 expressing LMP-1.

RESULTS AND DISCUSSION

3.1. Study of results obtained by serum protein electrophoresis on cellulose acetate
At basic pH, the majority of serum proteins are negatively charged. They migrate from the cathode to the anode and are separated into five fractions. Albumin which carries a significant negative charge migrates further toward the anode. Other proteins are a heterogeneous group of proteins, which are divided into four different sections: the α1 and α2 globulins, β and γ globulins.

The proteinogram obtained are generally very similar. They have a pace consistent with normal path, referenced in the literature. They do not show significant changes that may be characteristic of a particular syndrome. Almost all patients had normal proteinogram with proteinemia and concentrations of each protein fraction consistent with referential values used.

Table I: Variation of proteinemia and different protein fractions of patients

Samples	Proteinemia 60 - 80 g/l	Albumin 31- 50 g/l	α1 globulin 1 - 4 g/l	α2 globulin 7 - 13,5 g/l	β globulin 6 - 13,5 g/l	γ globulin 9 - 18 g/l
M01	64	33,9	1,7	8,7	6,7	13
M02	69	**28,9**	2,7	10,7	**13,8**	12,9
M03	**83**	38,4	2,8	12,5	11	**18,3**
M04	71	**27,4**	4,7	13,2	13,7	11,9
M05	**81**	39,5	3,9	12	11,7	13,9
M06	75	31,7	3,3	12,4	9,5	18
M07	**103**	35,8	3,3	**15,1**	**17,1**	**31,6**
M08	63	34,1	2,4	8,3	10,1	**8,2**
M09	70	31,1	2,3	9,7	10,3	16,5
M10	**58**	**27,1**	2,4	**6,8**	10,2	11,6
M11	80	43,3	3,2	9,8	11,3	12,5
M12	80	41,7	2,9	10,8	9,5	15
M13	**58**	**28,2**	2,2	10,1	7,3	10,2
M14	60	22,9	2,4	11,8	7,3	15,6
M15	**45**	20,4	2,1	**6,8**	**3,9**	11,7
M16	68	**29,4**	4	12,8	12,1	9,8
M17	75	32,2	4,5	9,3	10,5	18,4
M18	65	35,3	3,2	10,5	9,3	6,7
M19	69	31,4	5,1	10,6	8,5	13,3
M20	60	**27,2**	2,7	9,5	8,6	12

We have, however, found hyper-total protein in the patient M7 accompanied with increased concentrations of α2 fractions, β and γ globulins. We also demonstrated a hypo-proteinemia 45 g/l in the M15 patient with decreased

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levels of albumin and globulin β. As we have noted a significant hypoalbuminemia in subjects M14. The different variations of low magnitudes which have been identified are likely due to the presence of some artifacts during the analysis

The hyper-proteinemia seen in the M7 patient could be caused by a hyper-gammaglobulin polyclonal corresponding to an increase of all Ig classes following an immune response [12], as we also noted in this patient an increase in the concentration of the fraction of gammaglobulin with 31.6 g/l. The hypo-proteinemia are usually accompanied by hypo-albumin that could reveal the beginning of an inflammatory syndrome or caused by the increased of protein loss during gastrointestinal bleeding [13] [14].

The clinical symptoms of gastric cancer reported that patients most often suffer from localized pain in the abdomen, the pain testify to the affection of the stomach wall [15]. This complies with the work of WHICHER [13], which reports that the hypo-proteinemia can be caused by increased protein loss by leakage caused by digestive gastric bleeding. In addition, gastric cancer patients also complain of nausea and early satiety sensation which leads to a significant weight loss [16]. The work of Reif [14] confirmed that the patients' weight loss can be associated with hypo-proteinemia.

However, gastric cancer patients showed no profiles electrophoresis characteristics of the disease that could allow practitioners to a preliminary diagnosis of proteinemia since variations and concentrations of protein fractions are only isolated cases.

3.2. Study of protein profiles of serum proteins obtained by SDS-PAGE in the presence of β-mercaptoethanol
Human serum contains a variety of proteins with very different concentrations, which makes the difficulty of a specific study of these proteins and the desired assessing their role in the pathology studied [17].

The protein bands identified by SDS-PAGE in the presence of β-mercaptoethanol can be divided into major protein bands which are those that are almost always observed in normal human sera and minor bands that weakly or not appear at all in normal serum, but which may be characteristics of an electrophoresis profile referring to a given clinical situation [17].

The analysis of the electrophoresis profiles obtained by SDS-PAGE across the samples of sera analyzed both those of the healthy and those of gastric cancer subjects have in general a certain similarity and homogeneity concerning the presence of different bands and areas. We note, however, a disparity regarding the band 18 kDa which is present in 65% of patients with gastric cancer and only in 43% of healthy subjects. Furthermore, we found in 20% of sick individuals the presence of an additional band of 27 kDa.

The objective of the study of protein profiles obtained by SDS-PAGE is the determination of biomarkers, which would have the effect of distinguishing the occurrence of the pathological state. However, serum analysis is analytically difficult because of the high dynamic range of the proteins constituting it. An alternative to this problem can be considered in the study of low molecular weight serum proteome, which is the fraction of proteins and peptides from which the high molecular weight proteins have been removed [18] [19] [20], this requires other methods of analysis of serum proteins capable of depleting the serum of abundant proteins that would facilitate the detection of serum proteins of low abundance [21].

3.3.1. Revelation of the expression of LMP-1 by ELISA
The estimation of the LMP-1 value is made by comparing the observed optical density for each serum sample or protein extract with that observed for the negative control. Thus, all samples analyzed that have optical densities greater than that observed for the negative control are considered positive for LMP-1.

Analysis of the serum of gastric cancer patients showed that 50% of them were positive for the expression of LMP-1. Statistical analysis using the average compliance test was conducted to confirm the positive results compared to the negative control.

Concerning the LMP-1 rating in the protein extracts of gastric biopsies, the results were similar to those observed in the sera of patients with the disease. In the same manner as above, the average compliance test was used to confirm the results observed.

We also conducted an estimate of the expression of LMP-1 in healthy subjects' sera in order to check whether the LMP-1 expression was specific to gastric cancer patients. The results show that on all samples analyzed, only two of them showed superior to the negative control OD estimated at (0.184 ± 0.009). Thus, almost all of the healthy

subjects were negative for the expression of LMP-1. Compliance testing an average applied on samples from healthy individuals' sera confirms the results reported above.
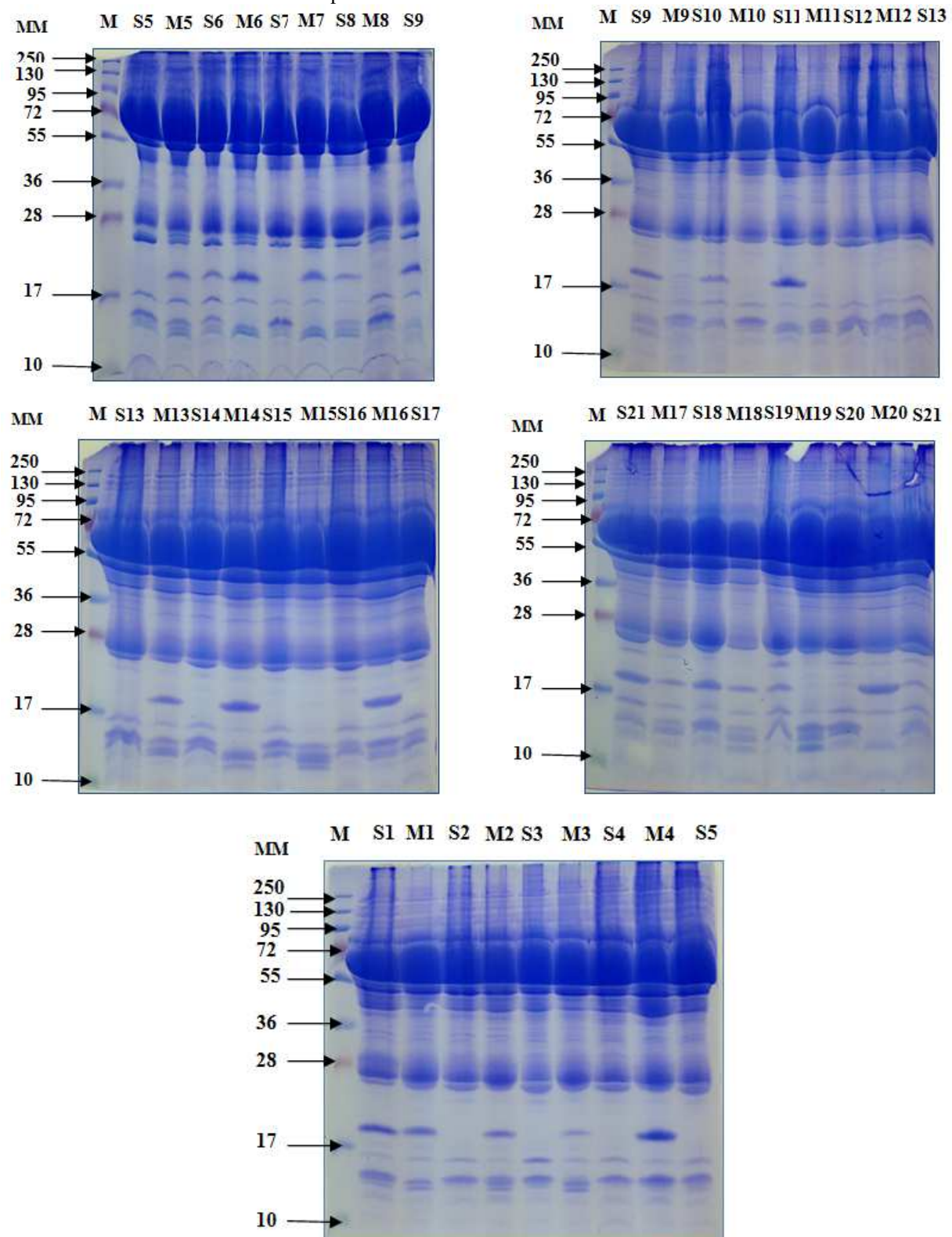

Figure 1: electrophoretic pattern obtained by SDS-PAGE in the presence of β-mercaptoethanol of healthy subjects serum samples: S1-S21 and patients with gastric cancer: M1- M20.
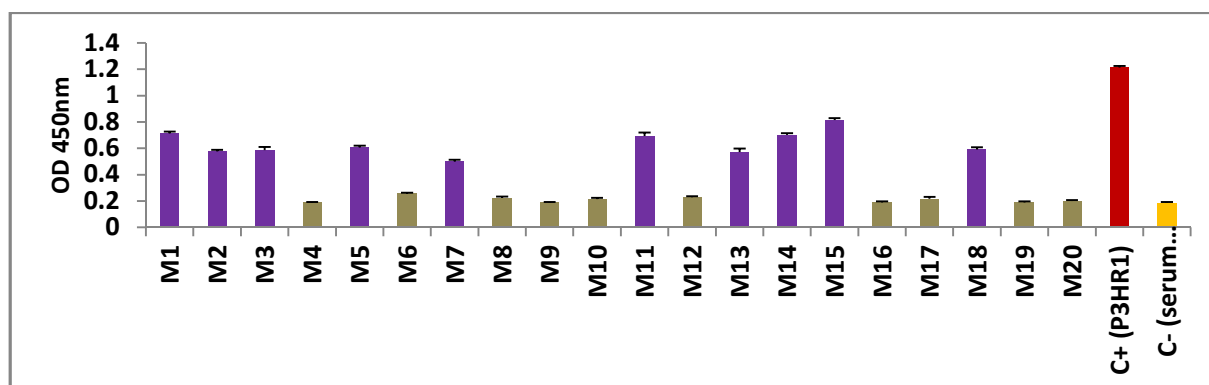
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3.3. Quantitative analysis of LMP-1 by ELISA

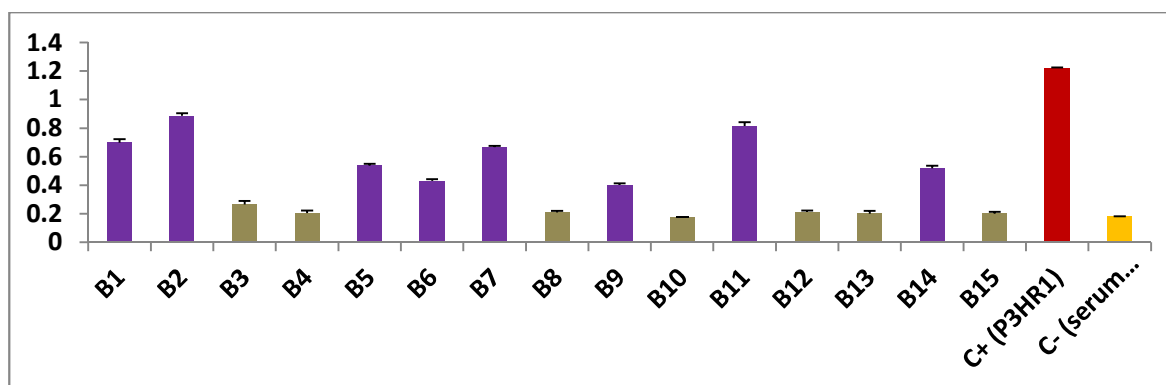**Figure 2a: Detecting the LMP1 with ELISA in serum of gastric cancer patients**

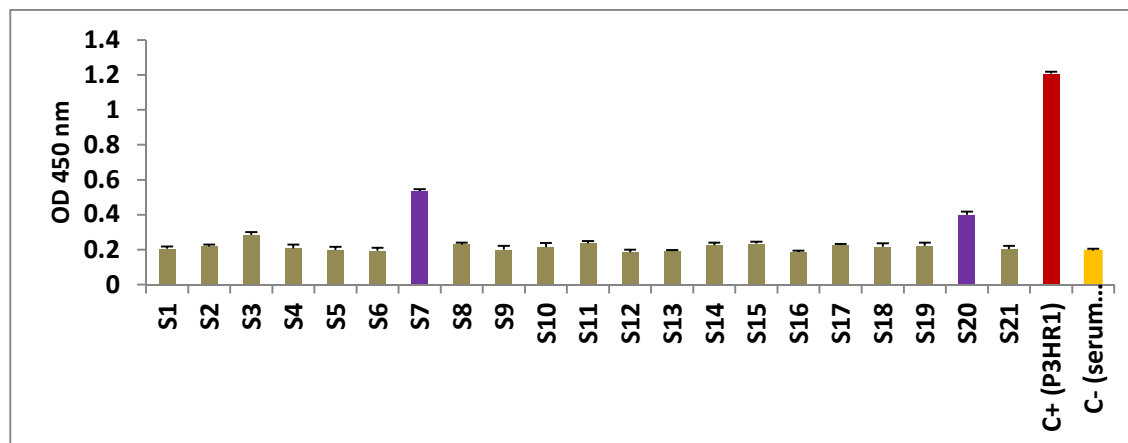**Figure 2b: Detecting the LMP1 with ELISA in biopsies of gastric cancer patients**

Figure 2c: Detecting the LMP1 with ELISA in the serum of healthy subjects

The revelation of the LMP-1 expression in protein extracts of gastric biopsies and in sera samples of gastric cancer subjects and its absence in the sera of healthy subjects confirms the association of gastric cancer and Epstein Barr virus, and a possible oncogenic role of LMP-1 in the installation cancer.

The association of gastric cancer with the Epstein Barr virus has been reported by several authors. The impact of this association varies according to the authors; it was estimated by [22] [23] [24], 6 to 16% of cases of gastric cancer identified. Our study found the association in nearly 50% of the cases studied. Since EBER1 is very abundant in cells infected by EBV in situ hybridization EBER1 (ISH) is the technique most used for the identification of gastric cancer associated EBV [25].

LMP-1 protein considered major oncogenic EBV with immortalizing and transforming properties [8], has not been so far associated with EBV positive gastric cancer. The work of FUKAYAMA [26]and those of MAEDA [27] show

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that the positive EBV gastric cancer has a type I latency expressing EBNA1, EBER, BART and sometimes (40% of cases) LMP2A protein and where expression of LMP-1 and EBNA2 is absent.

However, it is crucial to note that the work of MEIJ [28] showed that the majority of LMP-1 protein are localized in the Golgi apparatus. [29] reported that LMP-1 is associated with exosomes as vesicles and is secreted in this form in the epithelial cells. The LMP-1 protein originates from the level of the cellular machinery (nucleus and cytoplasm) and then it is conducted at the membrane to form transmembrane structure specific to it. It is likely that some of the LMP-1 protein complex to exosomes in order to activate certain signaling pathways such as NF-Kb. Thus, it is preferable to direct research of this protein towards its detection in exosomes. The anti LMP-1 antibody used in this study is the anti LMP1 S12 monoclonal that identifies LMP-1 in membranes as well as complexed with exosomes. Negative cases in the LMP-1 expression probably have gastric cancers negative EBV. It is true that oncologic role of EBV was highlighted on the carcinogenesis of gastric cancer, however, the virus is not the only factor implicated in the installation of this pathology. Studies suggest *Helicobacter pylori* may be an etiological factor in gastric cancer [30], together with dietary and environmental factors. These studies suggest that a diet rich in salt causes atrophic gastritis or dysplasia and promotes gastric carcinogenesis.

3.3.2. Relationship between the age of patient with gastric cancer and the expression of LMP-1 and between the evolution of gastric cancer and the expression of LMP-1

To highlight a possible relationship between the age of patient with gastric cancer and the expression of LMP-1 and between the evolution of gastric cancer and expression of LMP-1, we submitted all values LMP-1 obtained by the ELISA analysis by a non-parametric statistical test equivalent to variance analysis (ANOVA), which is the test of KRUSKAL and WALLIS and where the independent variable is either the age of the patient or the advancement of the disease.

The results of this test show that LMP-1 expression is independent of the age of the patient with gastric cancer. The EBV virus is transmitted through saliva during adolescence, but it is true that in developing countries, primary infection with EBV occurs early, usually in children under three years who are infected by their mothers [31]. This early infection does not seem to influence the oncogenic role of viruses in the gastric cancer installation. This study clearly demonstrated that there is no dependency between the age of the patient with gastric cancer and expression of LMP-1 and thus between patient's age and the association of pathology EBV. The work of Akiba [7] and those of Lee [25] suggested that EBV positive gastric cancer occurs in relatively young patients, but it is essential to note that meta-analyzes have not confirmed this observation.

The results also demonstrated that LMP-1 expression is independent of gastric cancer development, this means that the installation of EBV occurs very early in gastric epithelial cells and it persists during the disease. It obvious that EBV, as it is oncogenic, keeps its genome into the infected cell without killing it while protecting it from the attacks of the immune system [5], this could explain the findings of AKIBA [7] and FUKAYAMA [26] on the relatively good prognosis of EBV-positive gastric cancer compared with EBV-negative gastric cancer.

According to the result of this study, various studies are supportive of an etiologic role of EBV; these studies suggest that the virus infects early gastric epithelial cells allowing better proliferation, suggesting a role of viruses in carcinogenesis [32] [33]. As long as the LMP-1 is expressed early in patients with gastric cancer, this protein could probably serve as a biomarker for early diagnosis of EBV-positive gastric cancer.

CONCLUSION

This work allowed us to compare proteinograms of gastric cancer patients with those of healthy individuals. The only changes that spark interest are some hypo-proteinemia that can be caused by increased protein loss by leakage caused by gastric bleeding or by the loss of weight observed in individuals with gastric cancer. However, because they are not generalized to all patients, it cannot constitute a characteristic of the disease.

Previous investigations which are carried on the serum study showed difficulty identifying the different serum protein and highlight a characteristic proteome of pathology. The comparison of protein profiles of gastric cancer patients with those from healthy individuals allowed us to identify the presence of an additional band of 27 kDa in the former which does not exist in the latter. However, it is difficult to associate this protein band gastric cancer particularly as it is only present in 20% of cases. As for the 18 kDa protein that we find in 65% of patients and in 43% of cases in healthy people.

The detection of the expression of LMP-1 protein in serum and gastric biopsies of patients allows us to associate gastric cancer to EBV, especially since there is no expression of LMP-1 in the sera of healthy individuals. The

expression of LMP-1 regardless of patient age or the stage of development of gastric cancer may offer an alternative for diagnosis. Thus, LMP-1 appears to be a potential candidate for an early and reliable diagnosis of EBV-positive gastric cancer. Moreover, the LMP-1 detection in the sera of diseased individuals offers a tool that is simple regarding to the technique used, and especially non-binding for the patient often subjected to biopsy samples.

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