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IMMUNOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF EPITHELIAL MEMBRANE ANTIGEN IN CASE OF HEPATOCELLULAR CARCINOMA

[1]Attallah A.M., [1]Mohammed S.M. and [2]El-Waseef AM.
[1]R&D Dept., Biotechnology Research Center, New Damietta City, Egypt.
[2]Chemistry Dept., Faculty of Science,Mansoura Universty, Egypt.

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors in clinics. It is quite necessary and important to explore new approaches for early prognosis of HCC. The present study aimed to study the immunological and biochemical characterization of an epithelial membrane antigen associated with HCC. Serum samples from HCC patients (n=80) undergoing surgery and 20 serum samples from healthy individuals served as normal controls were used. The target epithelial membrane antigen (EMA) was purified from sera of HCC patients using electroelution technique and has been characterized. The purified EMA was characterized to be glycoprotein with molecular weight 130 kDa. Also, EMA was quantified in serum samples of 80 patients that were histopathologically proven to have HCC using ELISA. There was a highly significant difference between the value of EMA in HCC patients (8.9 ± 6.8 µg/ml) and that of normal individuals (1.6 ± 1.0 µg/ml) at ($P < 0.0001$). Accordingly, the estimation of serum EMA can be considered as a prognostic test for HCC.

INTRODUCTION

Hepatocellular carcinoma is now regarded as one of the major malignant diseases worldwide, with significant variations in its epidemiology. In Egypt the number of newly diagnosed patients with HCC increases annually. The prevalence of HCC is high in Nile Delta area, and is more common in males, especially in hepatitis C virus (HCV) patients. There are other risk factors that may be responsible for this high incidence, such as pollution, aflatoxins and use of insecticides [(1)]. Diagnosis by the imaging of small lesions is relatively inaccurate, whether by ultrasonography, computed tomography scanning, or Magnetic resonance imaging (MRI) [(2)]. Liver biopsy of small lesions is also insufficiently sensitive or specific [(3)]. The only serological marker widely used for the screening and diagnosis of HCC is alphafetoprotein (AFP) [(4)] but due to the limited sensitivity, particularly for small tumors, the usefulness of AFP measurement as a diagnostic test and surveillance tool for patients at risk for HCC has been questioned [(5)], EMA, known also as mucin 1 (MUC1), is a group of heavy glycosylated transmembrane proteins, present in a variety of glandular secretory epithelia and non-secretory epithelium. EMA can be demonstrated in most types of adenocarcinomas derived from secretory epithelia [(6)]. It has been found that MUC1 is aberrantly expressed in the form of misglycolization or incomplete glycolization in many tumor tissues like breast, stomach, and colon cancers. These abnormal MUC1 molecules reveal new protein epitopes or carbohydrate antigens, distributed all around the cancer cell surface, and may be recognized by the immune system as notable tumor-associated antigens [(7, 8)]. During the process of malignant transformation and invasion of tumor cells, the changes of MUC1 glycolization influence the biological behavior of tumor cells [(9)]. EMA is a valuable marker as a diagnostic and prognostic marker in liver carcinomas [(10)]. The characterization of an epithelial membrane antigen associated with HCC is, therefore, the aim of the present study.

SUBJECTS AND METHODS

A total of 80 Egyptian individuals (65 males and 15 females, age; 58 years ± 11 years) were included in the present study. All patients were diagnosed by Computed tomography (CT) scan of the abdomen using intravenous contrast agent and three-phase scanning. In most of patients there was a mass showed on abdominal CT scan. These patients showed increased serum levels of liver enzymes, reduced serum albumin, and increased serum billirubin. Examination of the liver biopsies showed HCC. Twenty serum samples from healthy individuals served as normal controls.

Purification of EMA by gel electroelution technique.

Epithelial membrane antigen was purified as described by [11]. Briefly serum proteins from patients with HCC and serum samples from healthy individuals were resolved using SDS-PAGE according to [12], and then transferred to nitrocellulose (NC) sheet according to the method of [13]. The NC membrane was blocked using 5 % (w/v) bovine serum albumin (BSA) dissolved in 0.05% M Tris-buffered saline (TBS), containing 200 mM NaCl (pH 7.4), rinsed in TBS, and incubated with epithelial membrane monoclonal antibody (mAb) (1 : 50) in 1% BSA dissolved in TBS with constant shaking. The NC membrane was washed three times, 15 min each, in TBS followed by incubation for 2 hrs with anti-mouse immunoglobulin G (IgG) alkaline phosphatase conjugate (Sigma) diluted 1: 500 in TBS. After washing, the NC membrane was exposed to alkaline phosphatase substrate [5-bromo-4-chloro-3-indolyl phosphate] (BCIP)/ nitroblue tetrazolium (NBT) in 0.1 M Tris buffer, pH 9.6; (Sigma) for 10 min and the reaction was stopped using distilled water. The specific band of target antigen appeared at 130 kDa. The target EMA (130 kDa) was cut and electroeluted from preparative polyacrylamide gels at 200 volts for 4 hrs in a dialysis bag (Sigma). After dialysis, the electroeluted antigen was concentrated using polyethylene glycol and 40% trichloroacetic acid (TCA), then centrifuged at 10,000 rpm for 15 min. The precipitate was washed twice using diethyl ether, to remove the excess TCA. The excess diethyl ether was removed by gentle

drying and the pellet was reconstituted in phosphate buffered saline (PBS), pH 7.2. The protein content of the electroeluted antigen was determined before it was stored at - 20 ˚C.

Biochemical characterization of the reactive epitope of the EMA*:*

The purified EMA was exposed to heat, chemicals, and proteolysis. The reactivity of EMA epitope towards epithelial membrane mAb was tested using ELISA after optimization of the reaction conditions. Polystyrene microtitre plates (Corning Life Sciences, Acton, AM) were coated with 50 µl/well of purified EMA (treated, untreated) in coating buffer (pH 9.6). After blocking, 50 µl/well of 1:75-dilution, in PBS with 0.05% (v/v) Tween 20 (PBS-T20), of a primary antibody epithelial membrane mAb were added to each well. Serum from healthy subjects was used as negative control. The plates were incubated at 37°C for 2 hours, washed, and then incubated, at 37° C for 1 hour, with anti-mouse IgG alkaline phosphatase (Whole molecule, Sigma) diluted 1:300 in PBS-T20 containing 0.2 % BSA. After washing, the substrate was added and the plates were incubated for 30 minutes at 37° C. Optical densities (O.D) were read at 490 nm using a micro-plate auto-reader (Metertech Inc. USA).The cut-off O.D for ELISA positivity was set as mean O.D plus three S.D for the sera from healthy subjects.

Dose curve of the purified EMA:

Dose-response curve for epithelial membrane antigen in the ELISA as a function of the concentration of antigen (µg /ml) in serum samples of controls and HCC patients was done. Serial concentrations from 37.2 to 1.2 µg /ml of the purified EMA were made and allowed to bind overnight to wells of ELISA plates. Epithelial membrane monoclonal antibody was then added. The antigen-antibody binding was allowed to proceed for 2 hours at 37 °C. Alkaline phosphatase-conjugated goat anti-mouse IgG, was added. The amount of coupled conjugate was determined by incubation with 1 mg/ml P-nitrophenyl phosphate in substrate buffer. The reaction was stopped by the addition of 3 M NaOH and the absorbance was read at 490 nm using a

microtiter plate reader. The cut-off level of ELISA above or below which the tested serum sample is considered positive or negative was calculated as the mean ELISA optical densities of 20 serum samples from controls + 3 standard deviations (cut-off = 2 µg /ml), and the serum samples of the selected positive HCC patients showed optical densities above the cut-off level, **(Fig. 1)**.

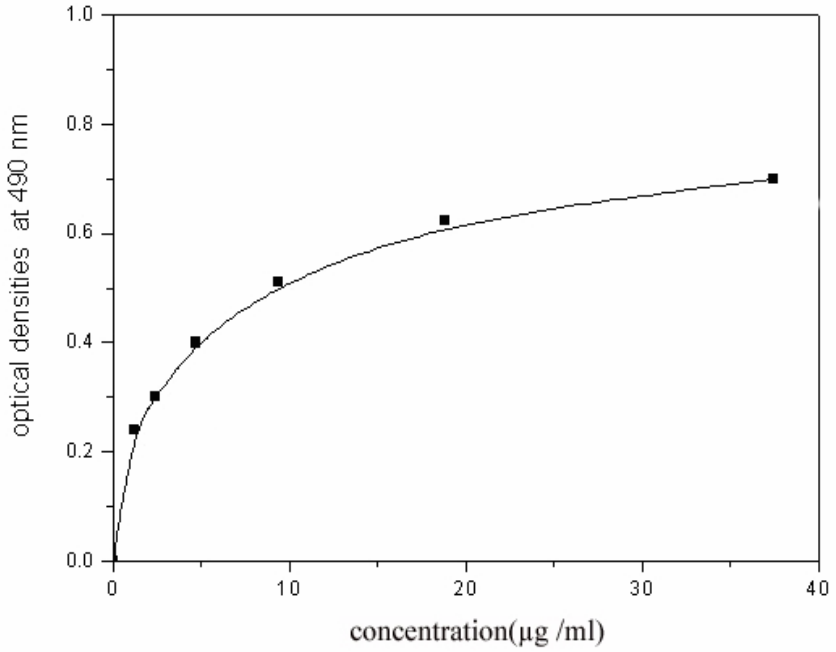


Fig. 1: Optical densities – concentration curve at 490 nm for purified 130 kDa EMA using ELISA.

RESULTS

Chemical nature of reactive epitope:

The reactivity of the purified EMA was lost after exposure to 56 ºC and higher degrees of temperature **(Fig. 2)**. Also the reactivity was lost after exposure to HCl, NaOH and periodate (**Fig. 3 a, b & 4)** but the reactivity was maintained after β-mercaptoethanol treatment **(Fig. 5)**. EMA precipitated with TCA showed high reactivity against epithelial membrane mAb in contrast to the supernatant which showed no reactivity (**Fig. 6).** When EMA was treated with a constant concentration of α-chymotrypsin at different time intervals (15, 30, 45, and 60 minutes), the reactivity was nearly lost after 15 minutes incubation and became constant thereafter **(Fig. 7).**

Quantitative detection of EMA in HCC patients using ELISA:

Serum samples of 80 patients who were histopathologically proven to have HCC were tested by ELISA for the detection of EMA compared to sera collected from 20 controls individuals. The average serum EMA concentration in HCC patients was 8.9 ± 6.8 μg/ml. However, in normal individuals the average was 1.6 ± 1.0 μg/ml**.** There was a highly significant difference between the value of EMA of HCC patients and that of normal individuals ($P < 0.0001$), **(Fig. 8).**

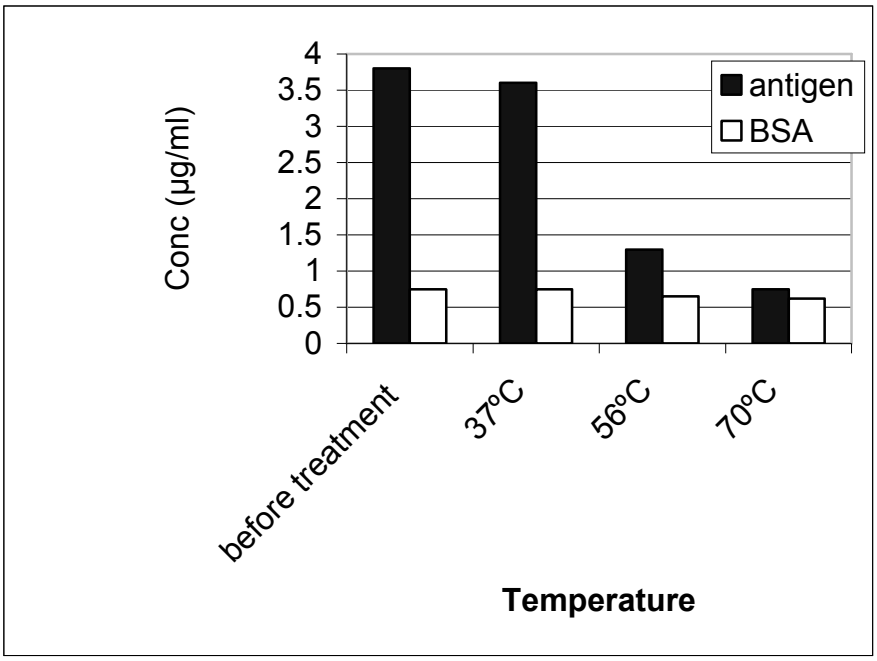
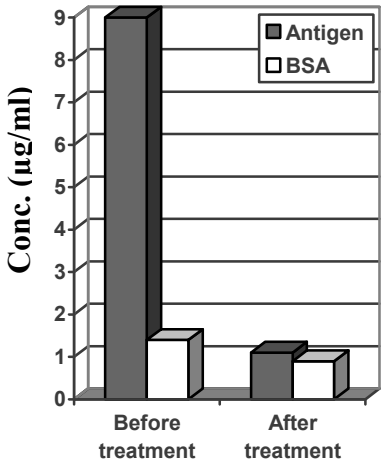
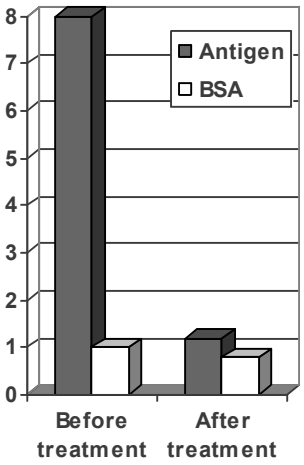


Fig. 2: Reactivity (conc., µg/ml) of 130 kDa purified EMA after incubation at 37°C, 56°C, and 70°C for 30 minutes. The reaction was tested against epithelial membrane mAb using ELISA.





Conc. (µg/ml)

(a) Effect of 0.2 M HCl **(b) Effect of 0.2 M NaOH**

Fig. 3: Reactivity (conc., µg/ml) of 130 kDa purified EMA after treatment with 0.2 M HCl (a) and 0.2 M NaOH (b). The reactivity was lost after acid and alkali treatments.

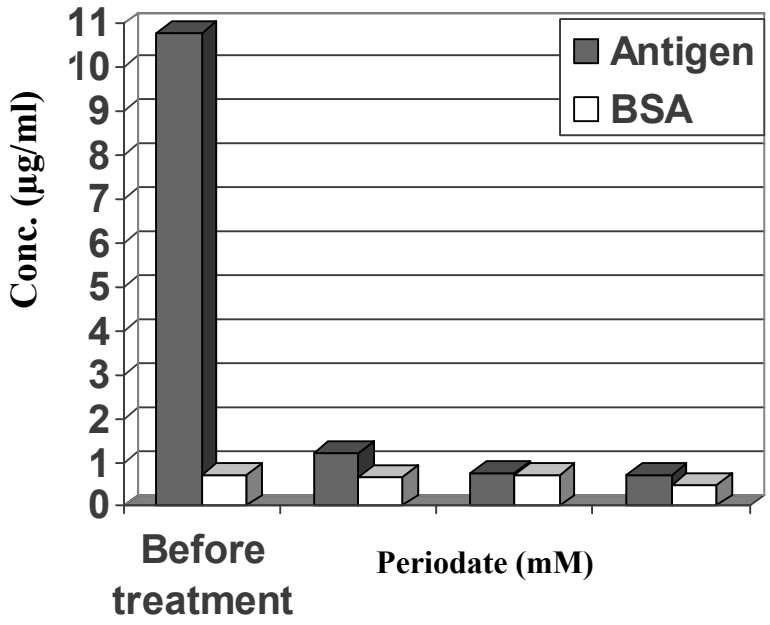


Fig. 4: Reactivity (conc., µg/ml) 130 kDa purified EMA after treatment with periodate. Purified EMA reactivity was completely lost after exposure to periodate at concentration of 10 mM.

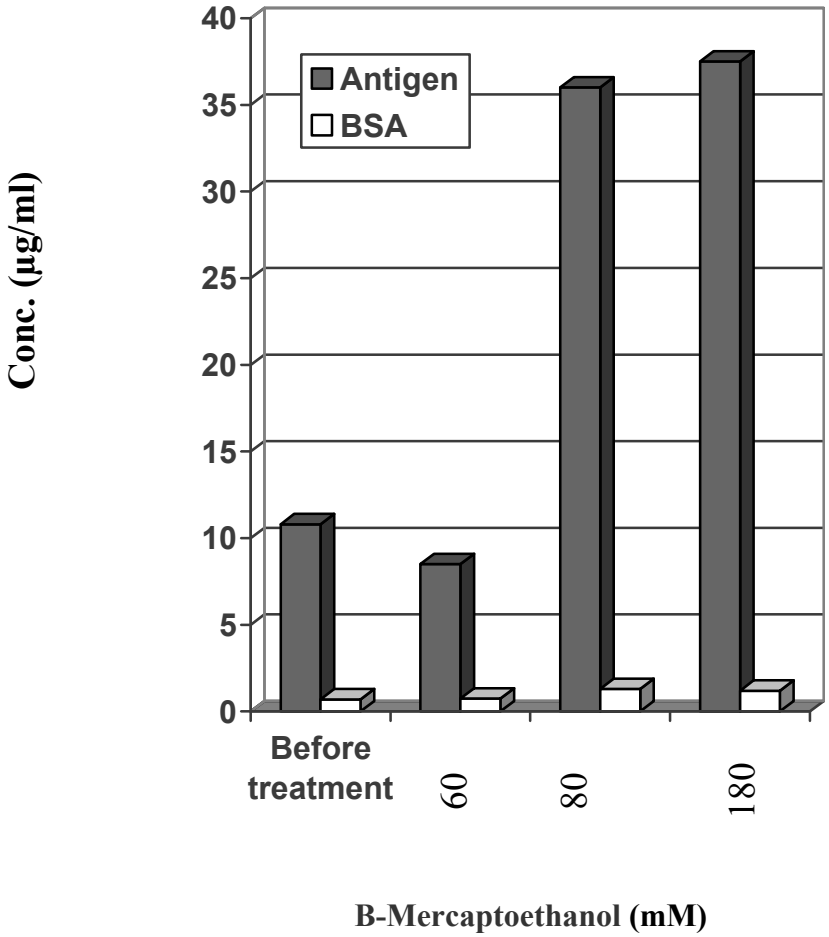


Fig. 5: Reactivity (conc., µg/ml) of the 130 kDa purified EMA after β-mercaptoethanol treatment. The purified EMA showed high reactivity towards epithelial membrane mAb, after β-mercaptoethanol treatment.

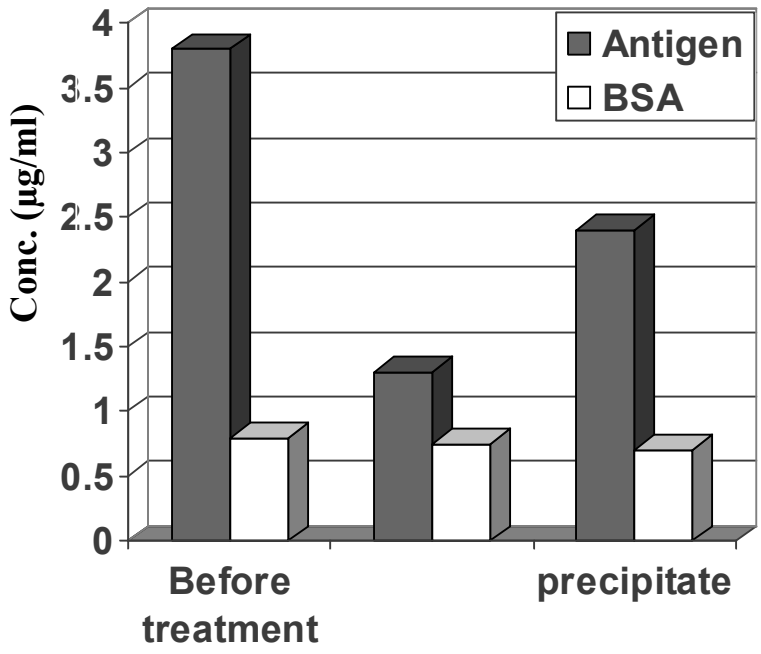


Fig. 6: Reactivity (conc. µg/ml) of the 130 kDa purified EMA after precipitation with TCA. The precipitate of purified EMA showed reactivity towards epithelial membrane mAb, while the supernatant showed no reactivity.

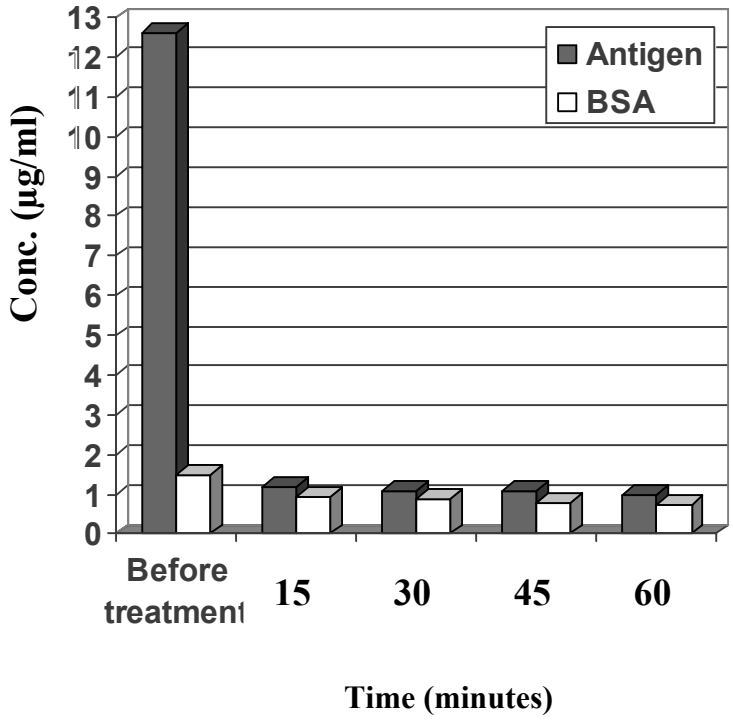


Fig. 7: Reactivity (conc., µg/ml) of 130 kDa purified EMA after incubation with α-chymotrypsin at 15, 30, 45, and 60 minutes. The reactivity was lost at all intervals.

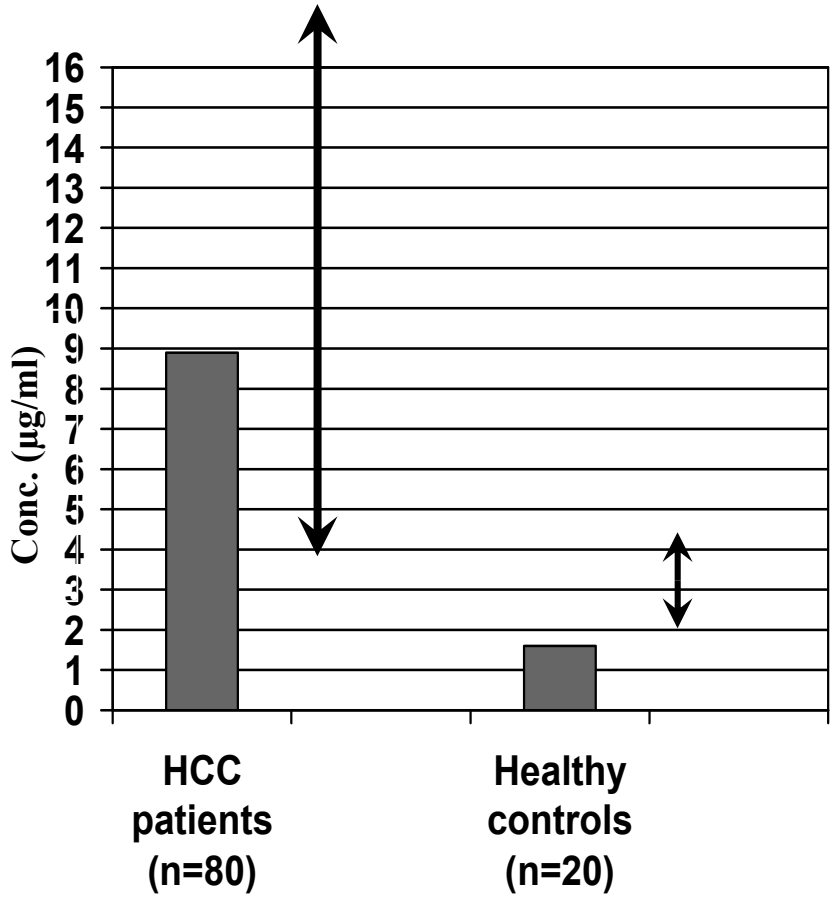


Fig. 8: Estimation of EMA in HCC patients using ELISA. There is a highly significant difference ($P < 0.0001$) between the average value of EMA of HCC patients and that of normal individuals.

DISCUSSION

The growth of HCC is characteristically silent in nature, which may delay the diagnosis for as long as 3 years from the time of development [14]. Diagnosis of HCC is usually easy in patients with a space-occupying lesion on ultrasonography or computed tomography, and serological AFP of more than 400 ng/ml [15]. In many cases, by the time, these conditions are met; HCC is incurable, as frequently the AFP is not diagnostically elevated. Diagnosis by the imaging of small lesions is relatively inaccurate, whether by ultrasonography, computed tomography scanning, or MRI [15]. Liver biopsy of small lesions is also insufficiently sensitive or specific [3]. Given the limitations of the AFP test, the search for more sensitive serological markers for HCC has continued. MUC1 expression is associated with primary liver cancer cell infiltration and metastasis as well. The possible mechanisms might be as follows. (1) E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, inter-cellular adhesion and is specifically involved in epithelial cell-to-cell adhesion. In cancers, decreased E-cadherin expression is one of the alterations that characterize the invasive phenotype, and the data support its role as a tumor suppressor, MUC1 promotes tumor metastasis by down regulating E-cadherin expression and its binding to beta-catenin [16]. (2) MUC1 acts as anti-cell adhesion molecules. High density of filamentous MUC1 molecules expressed on tumor cell surface might prevent binding between membrane-anchored ligands and corresponding receptors minimize intercellular interactions induced by integrin in extracellular matrix, thus facilitating cancer cell invasion [17]. (3) Sialyl Lewis epitopes on MUC1 molecules function as ligands to E-selectin in injured or inflammatory vascular endothelial cells, and facilitate tumor cell adhesion, infiltration, and metastasis [18]. In the present study, the purified EMA 130 kDa was characterized to be a glycoprotein whereas the antigen reactivity with epithelial membrane mAb in ELISA technique showed that temperatures below 56°C did not alter the recognition of the epitope. When the temperature was increased to 56°C, epithelial membrane mAb lost its reactivity

towards EMA (Fig. 2). The purified antigen was precipitated by 40% TCA, and the precipitated fraction showed high reactivity as the untreated antigen did. On the other hand, the soluble fraction did not show any reactivity with epithelial membrane mAb (Fig. 6). On the other hand the reactivity towards epithelial membrane mAb was lost after treating the purified EMA with 0.2 M NaOH, 0.2 M HCL (Fig. 3) or α-chymotrypsin (Fig. 7). The reactivity was also lost when the antigen was treated with oxidizing agents like sodium m-periodate (Fig. 4), which indicate that the antigen contains carbohydrates. But reactivity of purified EMA was recorded after treatment with reducing agents such as mercaptoethanol (Fig. 5).These findings confirm that the purified antigen is a glycoprotein. **Ormerod et al.,** *(19)* **stated** that carbohydrate forms the major component of EMA and the protein content of EMA is low.

Moreover, EMA was quantified in serum samples of 80 patients that were histopathologically proven to have HCC using ELISA against sera collected from 20 controls individuals. The mean serum EMA concentration in HCC patients was 8.9 ± 6.8 μg/ml. compared with that of normal individuals samples which was 1.6 ± 1.0 μg/ml**.** There is a highly significant difference between the value of EMA of HCC patients and that of normal individuals ($P < 0.0001$) (Fig. 8). **Hendrick et al.,** *(20)* described an ELISA method for EMA in normal population sera and the levels were in the range of 500 ± 125 ng/ml. In conclusion, the quantitation and characterization of epithelial membrane antigen may be of interest to be included in serodiagnosis of HCC.

CONCLUSION

A 130 kDa EMA was identified, isolated and characterized as glycoprotein. And EMA was quantified in serum samples of patients with HCC as a result it may be helpful in diagnosis of hepatocellular carcinoma.

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