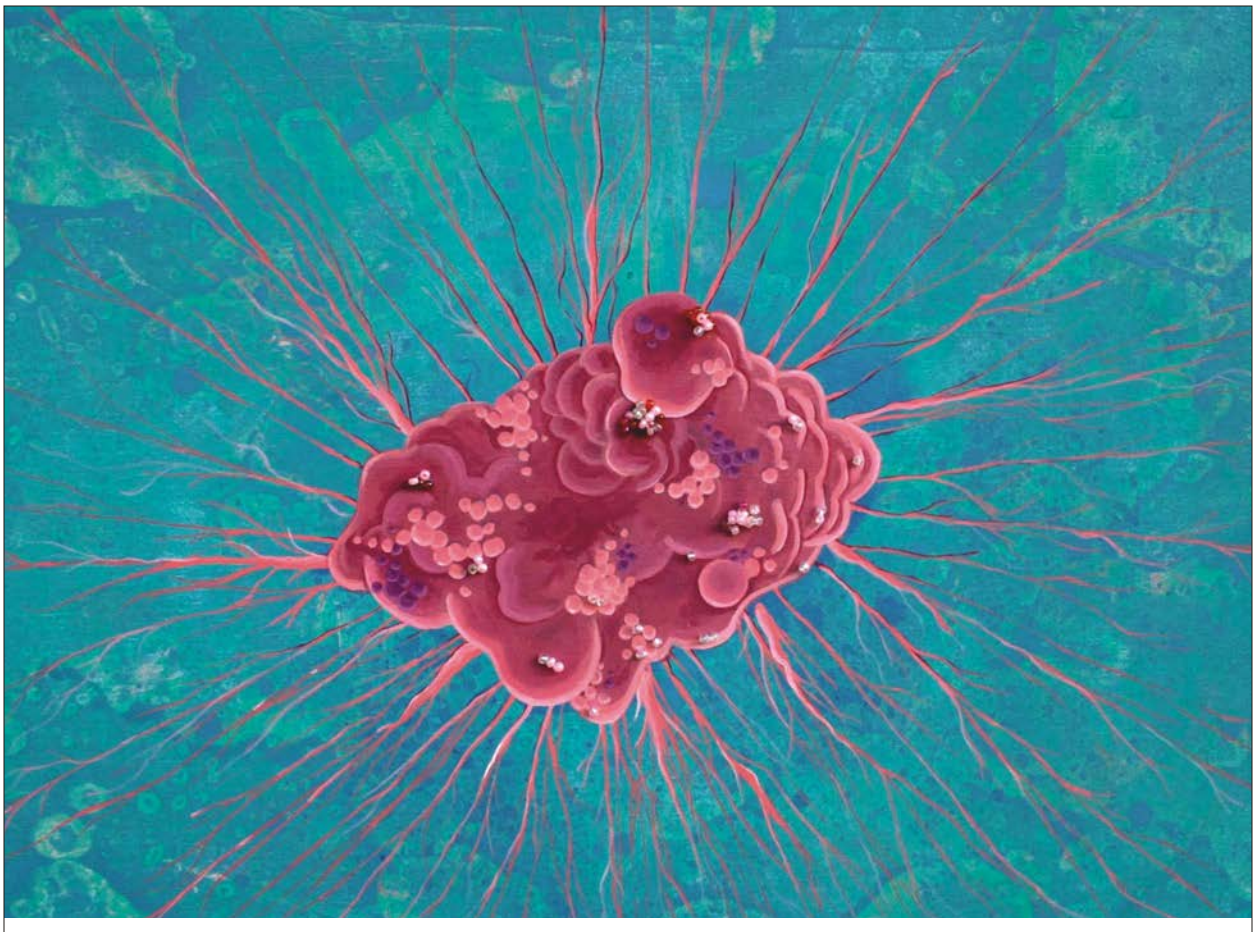

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Measurement of Serum Tumor Markers (Alpha-fetoprotein-CA 19.9) and DNA Ploidy in Liver Cirrhosis and Hepatocellular Carcinoma Patients

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Abstract: The present study aimed to investigate the measurement of serum tumor markers, Alpha-fetoprotein and CA 19.9 and its relation with DNA ploidy of biopsies from liver cirrhosis and hepatocellular carcinoma (HCC). Sera of patients with liver cirrhosis (n = 43) and HCC (n = 73) were tested. Alpha-fetoprotein (AFP) and CA 19.9 were measured according to instruction manual. DNA Flow cytometric analysis was done on liver biopsy from each patient. In liver cirrhosis patients, serum AFP and CA 19.9 were elevated, more than normal values, in 7.1 and 15.8%, respectively. Out of HCC patients, 65.75% showed abnormal elevation of serum AFP while CA 19.9 was found to be elevated abnormally in 48%. The combined measurement of AFP and CA19.9 markers demonstrated that 70% of HCC patients had abnormal elevation of serum concentration of AFP and/or CA 19.9. Aneuploid HCC showed a significant elevation ($p<0.001$) in tumor markers AFP and CA 19.9 compared with diploid HCC, but this relation was not clear in aneuploid liver cirrhosis. In conclusion, caution is needed in the interpretation of CA 19.9 results in the presence of liver dysfunction. Aneuploid HCC showed a significant elevation in AFP and CA 19.9 tumor markers, but this relation was not clear in aneuploid liver cirrhosis.

Key words: Ploidy, tumor markers, alpha-fetoprotein, CA 19.9, liver cirrhosis, hepatocellular carcinoma

INTRODUCTION

Tumor markers include cell surface antigens, cytoplasmic proteins, enzymes and hormones, are biochemical indicators of the presence of a tumor. In clinical practice, however, the term usually refers to a molecule that can be detected in plasma or other body fluids. Their main utility in clinical medicine has been as a laboratory test to support the diagnosis. Some tumor markers are also of value in determining the response to therapy and in indicating relapse during the follow-up period (Cotran *et al*., 1999). AFP is marker for hepatocellular carcinoma and germ cell carcinoma (Chan *et al*., 1999). AFP is also useful for determining prognosis and in the changes in clinical status. Elevated AFP levels after surgery may indicate incomplete removal of the tumor or the presence of metastasis. Falling or rising AFP levels after therapy may determine the success or failure of the treatment regiment. A significant increase of AFP in-patients considered free of metastatic tumor may indicate the development of metastasis (Chan *et al*., 1999). Carbohydrate antigen 19.9 (CA 19.9) is a tumor-associated antigen reported by Koprowski *et al*. (1981). CA 19.9 has been advocated as a sensitive and specific marker for the detection of pancreatic cancer (Steinberg *et al*., 1986). For reasons that remain unclear, serum CA 19.9 can often be found elevated in patients with liver diseases and of the billiary

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tract, both benign and malignant (Collazos *et al*., 1992; Marrelli, 2004). In the study of Kadayif *et al*. (1995), serum CA 19.9 levels investigated in patients with pancreatic adinocarcinoma and liver cirrhosis were to be elevated in 77.7% in-patients with pancreatic cancer and in 44% in-patients with liver cirrhosis. Also Nichols *et al*. (1994) demonstrated that the measurement of serum concentration of CA 19.9 is a promising test for detecting cholangiocarcinoma in patients with primary sclerosing cholangitis. Maestranzi *et al*. (1998) reported that the serum concentrations of CA19.9 were measured above the upper limit of the reference range (35 ku L^{-1}) in alcoholic liver disease (73%), primary sclerosing cholangitis (61%), primary biliary cirrhosis (60%), chronic hepatitis B (71%), chronic hepatitis C (84%), autoimmune hepatitis (36%) and hepatocellular carcinoma (54%). So studies of Maestranzi *et al*. (1998) and Giannini *et al*. (2000) indicated that caution is needed in the interpretation of CA19.9 results in the presence of liver dysfunction. From our recently study, (Attallah *et al*., 2006), we concluded that CA19-9 showed the best sensitivity for pancreatic cancer and AFP was the most sensitive tumor marker for HCC. In the present study, we measured the concentration of serum Alpha-fetoprotein and CA 19.9 and its relation to the changes in DNA ploidy in liver biopsies from patients with liver cirrhosis and hepatocellular carcinoma as detected by flow cytometry.

MATERIALS AND METHODS

Serum Samples

This study was carried out in Gastro-Enterology Center, Mansoura University during period of 1993- 1996. In the present study, sera of patients with liver cirrhosis (n = 43) and HCC (n = 73) were tested. Serum was separated, aliquoted in small volumes and stored at -20°C until use. Alpha-fetoprotein (AFP) (Eurogenetics, Belgium) and CA 19.9 (Sorin biomedica, Italy) were measured according to instruction manual. The absorbency values at 450 nm were determined using a spectrophotometer (El-311 microplate auto reader, Biotek, USA). A standard curve was obtained by plotting the absorbency values versus the corresponding standard values. The concentration of AFP or CA 19.9 in the patient samples was determined by interpolation from the standard curve.

Needle Liver Biopsies

Small needle liver biopsies were included in this study. One biopsy was taken each patient of liver cirrhosis or HCC. One biopsy was subjected to flow cytometry and the other to histopathology. Diagnosis was done according to clinical, ultrasound and pathological examinations.

Flow Cytometry

As reported in our previous studies (Attallah *et al*., 1999; El-Sayed *et al*., 2004) briefly, single cell suspension was prepared by mechanical dissociation of the fresh biopsy specimen in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) followed by filtration through a piece of fine nylon mesh (45 μm pore size) and centrifugation to remove debris and cell clumps. Cells were permeabilized with Triton X-100 (Sigma) followed by staining using Propidium Iodide (Sigma) as a DNA-Specific fluorochrome. Flow cytometric analysis was performed with a coulter EPICS profile II flow cytometer (Coulter Corp., Hialeah, Fl., USA), configured with a 488 nm argon ion laser. Peripheral blood lymphocytes were used as an external stander for tissue material. A total of 20,000 events per sample were acquired. DNA histograms were analyzed using cytological software (Coulter Corp., Hialeah, Fl., USA). Histogram showing only one G0/G1 peak is considered as diploid cells (normal) and that showing two distinct peaks is considered as aneuploid cells. DNA index was measured by determination of the ratio of DNA content of aneuploid peak to the DNA content of the diploid peak. DNA aneuploid population has DNA index higher than 1.1 N in 10% or more of the nuclei.

RESULTS

In liver cirrhosis patients, 7.1% (3/43) showed elevation of AFP. CA19.9 was elevated in 15.8% of liver cirrhosis. Out of 73 HCC patients, 48 (65.75%) showed abnormal elevation of AFP (over 10 IU mL^{-1}). Of them, 15 cases (31.2%) showed elevation more than 100 IU mL^{-1}. CA 19.9 was elevated abnormally (more than 37 IU mL^{-1}) in 29/60 (48%) of HCC patients, four of them (13.8%) showed elevation more than 300 IU mL^{-1}, (Table 1). Considering at least one of the two markers elevated indicated abnormal elevation, 70% indicated abnormal elevation of tumor markers of AFP and/or CA 19.9. The serum concentration of AFP in liver cirrhosis was not as that in HCC patients. The range of AFP concentration in liver cirrhosis was 15-20 U mL^{-1}, but in HCC patients the concentration was 75 U mL^{-1} - >300 U mL^{-1}. Out of 43 liver cirrhosis patients, 27 patients (62.8%) showed DNA diploid and 16 patients (37.2%) showed DNA aneuploid as measured by flow cytometry, (Fig. 1). Of 27 patients with diploid liver cirrhosis, one patient (3.7%) had elevation in AFP and 3 (11.1%) had elevation in CA19.9. Out of aneuploid liver cirrhosis, one (3.7%) had elevated AFP and 4 (14.8) patients out of 27 aneuploid liver cirrhosis had elevated CA 19.9. Out of 48 HCC patients who had elevation in AFP (>10 IU mL^{-1}), 36 (75%) cases were aneuploid and 12 (25%) cases were diploid. Out of these 48 HCC cases, 15 (31.25%) cases had elevation in AFP more than 100 IU mL^{-1} and all of these 15 cases (100%) were aneuploid, (Table 2). CA 19.9 was found elevated abnormally (more than 37 IU mL^{-1}) in 29/60 (48%) of HCC patients, four of them showed elevation more than 300 IU mL^{-1}. Out of 29 HCC cases who had elevation in CA 19.9 (more than 37 IU mL^{-1}), 20/29 (69%) cases were aneuploid and 9/29 (31%) were diploid). Out of these 29 cases, 4 cases had elevation in CA 19.9 more than 300 IU mL^{-1} and all of these 4 (100%) cases were aneuploid. On the other hand, aneuploid HCC showed a significant ($p<0.001$) elevation in tumor markers alpha-fetoprotein and CA 19.9 in compared with diploid HCC. But this relation was not clear in aneuploid liver cirrhosis (Table 2).

Table 1: Elevation of alpha-fetoprotein and CA 19.9 in serum samples from patients with liver cirrhosis and hepatocellular carcinoma

Tumor marker	Liver cirrhosis (%)	HCC (%)	p-value
Elevated alpha-fetoprotein			
More than 10 IU mL^{-1}	7.1	65.75	p<0.001
More than 100 IU mL^{-1}	0.0	15.00	p<0.001
Elevated CA 19.9			
More than 37 IU mL^{-1}	15.8	48.00	p<0.001
More than 300 IU mL^{-1}	0.0	13.80	p<0.001

Alphafetoprotein and CA 19.9 were measured by ELISA , p<0.001: highly significant

Table 2: Elevation of Alpha-fetoprotein and CA 19.9 in diploid and aneuploid liver cirrhosis and hepatocellular carcinoma patients

Tumor marker	Liver cirrhosis (%)		HCC (%)	p-value
Elevated alpha-fetoprotein				
More than 10 IU mL^{-1}				
Diploid	3.7		25.0	
Aneuploid	3.7	NS	75.0	p<0.001
More than 100 IU mL^{-1}				
Diploid	0.0		0.0	
Aneuploid	0.0		100.0	p<0.001
Elevated CA 19.9				
More than 37 IU mL^{-1}				
Diploid	11.1		31.0	
Aneuploid	14.8	NS	69.0	p<0.001
More than 300 IU mL^{-1}				
Diploid	0.0		0.0	
Aneuploid	0.0	NS	100.0	p<0.001

Alphafetoprotein and CA 19.9 were measured by ELISA, DNA content (Diploid and aneuploid) was measured by flow cytometric analysis, NS: Non Significant, p<0.001: Highly Significant

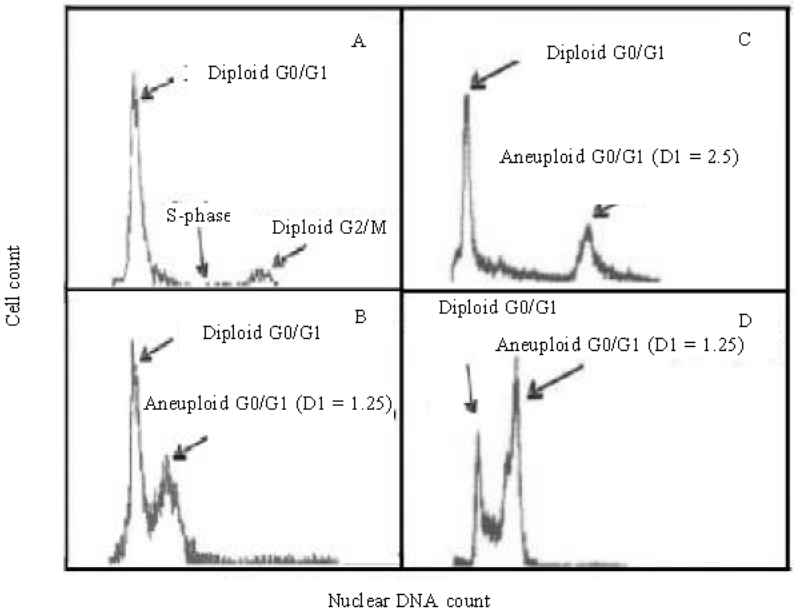

Fig. 1: Flow cytometric analysis of total DNA content (Ploidy) of liver biopsies form (A) normal liver, (B) liver cirrhosis, (C, D) hepatocellular carcinoma. DI: DNA Index

DISCUSSION

Tumor markers in particular CA 19.9 and Alpha-fetoprotein (AFP), have aided detection of pancreatic and hepatocellular carcinoma, respectively. In addition to the association of the CA 19.9 epitope with pancreatic neoplasia, this marker has also been found in the sera of patients with tumors arising at a variety of sites. The antigen has been found in 40 to 80% of carcinomas from gall bladder, stomach, pancreas and colon (Jalanko *et al*., 1984). Other studies demonstrate that CA 19.9 may be helpful in diagnosing pulmonary sequestration (Nakamura *et al*., 1997) and in urothelial cancer, especially in low grade cancer because its urinary level is high and it is more sensitive than urinary cytology (Noto *et al*., 1997). In benign diseases there are also frequent false positives, especially in pancreatic and hepatobiliary diseases, although usually at concentrations lower than in cancer patients (Paganuzzi *et al*., 1988). In the present study, CA 19.9 was found to be elevated abnormally in 15.8% and 48% of liver cirrhosis and HCC patients and, respectively. Fabris *et al*. (1995) reported that increased levels of CA 19.9 are common in patients with advanced liver disease, both benign and malignant. Also, Giannini *et al*. (2000) showed that caution should be used when evaluating CA19-9 in cirrhotic patients with cholestasis, since false positive results may occur. Alpha-fetoprotein (AFP) is the well-known gold standard tumor marker of hepatocellular carcinoma (HCC) (Tamura *et al*. 1998, Yeh and Chen, 2004). Sato *et al*. (1998) demonstrated that AFP staining of tissue specimens obtained by fine needle biopsy is useful in the histologic diagnosis of HCC. McIntire *et al*. (1975) reported extraordinarily high positive rates of AFP (>40 ng mL^{-1}) for pancreatic (23 to 24%) and biliary tract (25%) cancers. It is generally accepted that a considerable number of patients with acute and chronic hepatitis and cirrhosis without malignancy show slight but significant elevation of AFP (Lehmann and Wegener, 1979). The recognition of raised serum levels of AFP is clinically important because AFP positive patients with chronic hepatitis and cirrhosis have a high risk of liver related death or development (Hirai, 1987). Khalifa *et al*. (1999) demonstrated that AFP at an optimal cut-off value of 100 ng mL^{-1} and TPA at 160 U L^{-1} showed the highest sensitivity and specificity in detecting liver metastasis (100 and 87% for AFP; 100 and 54% for TPA, respectively) and so they indicated that

the combined assay of AFP and TPA resulted in a better discrimination of HCC among patients with hepatic focal lesions. In the present study, HCC and liver cirrhosis patients showed abnormal elevation of serum alpha-fetoprotein 65.75 and 7.1%, respectively. The present results agree with Lehmann and Wegener (1979); Sadek *et al*. (1997) and Tamura *et al*. (1998). There is difference in the range of serum concentration of AFP or CA 19.9 in liver cirrhosis and HCC patients and this is in agreement with Paganuzzi *et al*. (1988). Wiercinska-Drapalo *et al*. (1997) measured the serum alpha-fetoprotein concentration in patients with different stages of liver cirrhosis demonstrated through scored child-Pugh classification. AFP concentration revealed a significant positive correlation with score value. Ng *et al*. (1994) found that, the diploid tumors had serum alpha-fetoprotein levels increased to greater than 500 mg mL^{-1} more frequently than did the aneuploid tumors ($p<0.0001$). In the present study, 75 and 69% of aneuploid HCC have elevation in alpha-fetoprotein (more than 10 U mL^{-1}) and CA 19.9 (more than 37 U mL^{-1}), respectively which is more frequently than diploid HCC, although no correlation between elevated concentration of alpha-fetoprotein or CA 19.9 with DNA Index. Hamazaki *et al*. (1993) found no correlation between DNA ploidy pattern and serum alpha-fetoprotein levels. However in the study of Ng *et al*. (1994), aneuploid was observed in 46.9% of HCC specimens but in our study, aneuploid found in 78.6% of HCC tumor lesion and in 42.9% of HCC residual liver tissues. In conclusion, caution is needed in the interpretation of CA19.9 results in the presence of liver dysfunction. Aneuploid HCC showed a significant elevation in tumor markers alpha-fetoprotein and CA 19.9, but this relation was not clear in aneuploid liver cirrhosis. Also caution should be used when evaluating CA19-9 in cirrhotic patients, since false positive results may occur.

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