Seroprevalence of Glypican-3 (GPC3) in patients with pancreatic, gastric and esophageal cancers

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ABSTRACT: Glypican-3 (GPC3) is a member of the glypican family of heparan-sulfate proteoglycans (HSPGs), which are linked to the cell surface through a glycosyl-phosphatidylinositol anchor. The present work was suggested to use the ELISA technique, based on monoclonal antibody (GPC3 – mAb), to identify and quantify the GPC3 levels in serum samples from patients with pancreatic, stomach and esophageal cancers. Obtained results revealed that there was a highly significant difference in GPC3 levels between pancreatic cancer patients and healthy individuals. Positive GPC3 samples were detected in 35 out of 40 pancreatic cancer samples with a sensitivity 87.5% and specificity 100 % compared to healthy individual samples while the overall accuracy of the test was 90.7%. Results revealed also that there was no significant difference in the levels of serum AFP between pancreatic cancer patients and controls. A significant difference in serum GPC3 levels was also found between pancreatic cancer patients and gastric cancer patients. GPC3 protein might be over expressed in patients with pancreatic cancer with subsequent release into blood circulation enabling its assay using the noninvasive ELISA technique that optimized based on specific monoclonal antibody (GPC3 – mAb). In conclusion; GPC3 is more sensitive tumor marker and can be used as a diagnostic and prognostic marker for pancreatic cancer and it is preferred as a tumor marker than the classical tumor marker AFP.

1. Introduction

Tumor markers are biologic or biochemical substances produced by tumors and secreted into blood, urine, other body fluids or body tissues of some patients with certain types of cancer in amount higher than normal. A tumor marker may be produced by tumor itself, or by the body in response to the presence of cancer or certain non-cancerous conditions (Rustin et al., 2002). Tumor markers have been used in several settings in cancer patients, including screening measures (Chan and Schwartz., 2002), differentiating malignant from benign lesions, monitoring the response to treatment, and detecting recurrences (Maehara et al., 2006).

Glypicans are a family of heparan-sulfate proteoglycans (HSPGs) that are linked to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (Filmus and song 2000). Six members of this family (GPC1 to GPC6) have been identified in mammals (Paine-Saunders et al, 1999). As a member of the glypican family, glypican-3 (GPC3) encodes cell-surface heparan-sulfate proteoglycans, and is frequently up regulated in hepatocellular carcinoma (HCC) (Peters et al., 2005), ovarian cancer (Lin et al, 2005), breast cancer (Murthy et al., 2006) and melanoma (Nakatsura and Nishimura, 2006) as well as gastric cancers (Zhu et al, 2002).

The uses of tumor markers are numerous. However, regardless of the type of tumor marker, in order for a marker to be measured for routine implementation, the marker ultimately should impact on clinical management of the malignant disease either by improving patient outcome or quality of life or by lowering costs of care (Hayes et al, 1996). The aim of this study is to detect the prevalence of GPC3 level in serum samples from selected patients with pancreatic, stomach and esophageal cancers, to evaluate its clinical significance and to determine the associations between serum GPC3 and other tumor markers such as -fetoprotein (AFP).

2. Materials and Methods

This study was conducted in the Gastroenterology Center, Mansoura University from November 2006 till March 2009. Serum samples were obtained from separation of blood from patients with pancreatic (27 males and 13 females, age 39 – 84 years), esophageal (4 males and 4 females, age 25 – 60 years), and stomach (16 males and 8 females, age 27 – 70 years) cancers. Fourteen serum specimens from 10 males and 4 females healthy individuals (age 33 – 66 years), were also analyzed as controls. Serum samples were kept frozen at – 20 ^{o}C till analyzed for GPC3, AFP

and some routine analyses as ALT, AST and Bilirubin.

ALT & AST assays and bilirubin estimations were performed for all serum samples by standard automated methods using an autoanalyzer (Hitachi 902 autoanalyzer S.N. 1048008). Anti-HCV antibodies and HBsAg in sera of patients and healthy controls were determined by the rapid chromatographic immunoassay for the qualitative detection of hepatitis markers according to the method of Wilber (1993).

Serum GPC3 levels of patients and healthy individuals were determined by commercially available enzyme linked immunosorbent assay (ELISA) kit according to the manufacture's protocol (GPC3- - mouse EIA kit, Assay Design Inc, Ann Arbol MI.USA) with Glypican-3 (1G12) sc-65443, Lot # 12107 mouse monoclonal IgG, Santa Cruz Biotechnology, Inc.

After optimization of the reaction conditions, polystyrene microtiter plates were coated with 50 μl/well of each serum sample diluted 1:1000 in carbonate/bicarbonate buffer (pH 9.6). The plates were incubated overnight at room temperature and washed three times using 0.05% (v/v) PBS-T20 (pH 7.2) and then incubated for 1 hr at room temperature with 200 μl/well of 0.2% (w/v) non-fat milk in carbonate/bicarbonate buffer (pH 9.6). After washing, 50 μl/well of mouse monoclonal antibody GPC3, diluted 1:500 in PBS-T20, were added and incubated at 37°C for 2 hrs. After washing, 50 μl/well of anti-mouse IgG peroxidase conjugate (Sigma), diluted 1:1500 in PBS-T20, were added and incubated at 37°C for 1 hr. Excess conjugate was removed by extensive washing and the amount of coupled conjugate was determined by incubation with 50 μl/well tetramethyl benzidine (TMB) (KPL Cat No. 50-76-00) at 37°C. After appropriate time, the absorbance was read at 490 nm using microplate autoreader (Bio-Tek Instruments. WI, USA). Cutoff level of ELISA above or below which the tested sample is considered positive or negative was calculated as the mean concentration of serum samples from healthy individuals + 2SD.

The solid phase enzyme-linked immunosorbent assay was used for quantitative determination of AFP in human serum. A monoclonal anti-AFP antibody conjugated to horseradish peroxidase (HRPO) was in the antibody-enzyme conjugate solution. The test sample was allowed to react first with the immobilized rabbit antibody for 30 minutes. The monoclonal-HRPO conjugate was then reacted with the immobilized antigen for 30 minutes at room temperature resulting in the AFP molecules being sandwiched between the solid phase and enzyme linked antibodies. TMB solution was added and the

plates were incubated for 20 minutes resulting in the development of a blue color. The color development was stopped with the addition of stop solution changing the color to yellow. The absorbance was measured spectrophotometrically at 450 nm on microtiter plate reader (Sell, 1990). The minimum detectable concentration of AFP by this assay is estimated to be 2.0 ng/ml. Statistical measures were analyzed by the paired or unpaired student's t test. *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

As shown in table (1), serum AST and ALT were significantly (*P = 0.001* for AST and *P = 0.006* for ALT) higher in pancreatic patients than their values in controls.

Serum bilirubin level of pancreatic cancer patients was significantly higher than that of controls (*P = 0.0024*). All patients and healthy controls were negative for HBsAg while 22 out of 40 pancreatic, 9 out of 40 stomach and 2 out of 8 esophageal cancer patients and 1 out of 14 of the controls were positive for HCV antibodies.

Only four out of 40 (10 %) were positive for AFP in pancreatic cancer patients and level of serum AFP ranged from 5 to 139 ng/ml. The levels of AFP in the three groupd of patients are not statistically different from the level of the control group.

As shown in Table (2), serum GPC3 levels in pancreatic cancer patients ranged from 0.127 – 0.456 and averaged of 0.31 $\pm$ 0.093. This average is significantly *(P<0.0001)* higher than that of healthy individuals. The cut off value for the GPC3 in patients with pancreatic cancer was 0.132 which is 2 SD above the average of the healthy individuals. Therefore, serum GPC3 levels above 0.132 were considered positive. Accordingly, 35 out of 40 (87.5 %) pancreatic cancer patients were positive for serum GPC3. The specificity of GPC3 was calculated (according to the healthy individuals) and found to be 100 % and the overall accuracy of the test was 90.7% (Figure 1).

The mean serum levels of GPC3 for stomach , and esophageal cancer patients were highly significant (*P<0.0001* for stomach and *P<0.0001* for esophageal cancers) with respect to the mean control value (Table 3). Our result showed that 18 out of 24   (75 %) patients with stomach cancer and 5 out of 8 (62.5 %) patients of esophageal cancer were positive for GPC3. The main of serum GPC3 level in male pancreatic cancer patients was statistically significant (P< *0.0001*) than that of male stomach one. Also, the difference between the level of GPC3 in female pancreatic patients was statistically significant (P<0.0001) than that of male stomach one. In other

side, there was a highly significant (*P=0.0062 and P= 0.0202*) difference between the mean of serum GPC3 value in both male and female pancreatic cancer patients and female stomach one.. In addition, pancreatic cancer patients with normal liver function tests also had significantly (*P<0.0001*) higher serum level of GPC3 than both stomach and esophageal ones

Table (1): Clinical and biochemical characteristics for patients and healthy individuals

Parameter	Control (n = 14)	Pancreatic (n= 40)	Stomach (n= 24)	Esophagus (n= 8)
Age (yr)	43.57 $\pm$ 10.04	56.95 $\pm$ 9.2	49.92 $\pm$ 11.59	44.11$\pm$ 13.85
Sex M/F	10/4	27/13	16/8	4/4
HCV +/-	1/13	22/18	9/15	2/6
HBV +/-	0/14	0/40	0/24	0/8
Bil. (mg%)	0.81 $\pm$ 0.27	*10.15 $\pm$10.89	**2.65 $\pm$ 6.0	***1.59 $\pm$ 2.21
ALT (IU/l)	26.57 $\pm$ 8.59	©58.18 $\pm$ 40.28	©© 59.25 $\pm$ 75.1	©©© 31 $\pm$ 10.5
AST (IU/l)	30.64 $\pm$ 7.72	72.3 $\pm$ 44.2	46.29 $\pm$36.54	29.38 $\pm$ 9.7
AFP (ng/ml)	10.25 $\pm$ 3.92	® 17.9 $\pm$ 22.5	®® 12.29 $\pm$ 5.88	®®® 11.75 $\pm$ 4.86

n = number, ALT: alanine aminotransferase, AST: aspartate aminotransferase, NS: Not significant,HS: highly significant

* P = 0.0024 HS   ** P= 0.2675 NS   *** P= 0.2022 NS
© P = 0.0065 HS   ©© P=0.1155 NS   ©©© P= 0.296 NS
   P = 0.001 HS   P = 0.1244 NS   P = 0.739 NS
® P = 0.185 NS   ®® P = 0.231 NS   ®®® P = 0.423 NS

Table (2): Prevalence of serum glypican-3 (GP3) in our patients and healthy controls

Group	No.	GP3 (O.D) Range Mean $\pm$ SD	GP3 status	
			+Ve > 0.132 (O.D)	-Ve $\leq$ 0.132 (O.D)
Healthy control	14	0.118 – 0.131 0.124 $\pm$ 0.004	0	14
Pancreatic cancer	40	0.127 -0.456 0.31 $\pm$ 0.093	35	5
Stomach cancer	24	0.116 – 0.289 0.183 $\pm$ 0.05	18	6
Esophagus cancer	8	0.115 – 0.211 0.166 $\pm$ 0.04	5	3

Cut off value = Mean of healthy control + 2x SD
Cut off = 0.124 + 2 (0.004) = 0.124 + 0.008 = **0.132**
$\leq$ 0.132   is considered negative
> 0.132   is considered positive
* when comparing the healthy control with pancreatic cancer p<0.0001, with stomach cancer p =0.0002 and with esophagus cancer p= 0.0008
 O.D: optical density

Table (3): prevalence of serum GPC3 levels in male and female patients

Parameter	GPC3 *(OD)*	*Stomach Mean $\pm$ SD	Stomach M Mean + SD	Stomach F Mean + SD	**Esophagus Mean + SD	Esophagus M Mean + SD	Esophagus F Mean + SD
Pancreatic	0.31 $\pm$ 0.1	0.2 $\pm$0.06			0.17 $\pm$ 0.04		
Male	0.31 $\pm$ 0.09		@ 0.15 + 0.056	0.21 $\pm$0.05		® 0.16 $\pm$ 0.033	0.18 $\pm$0.04
Female	0.311 $\pm$ 0.1		# 0.15 + 0.056	© 0.21 $\pm$0.05		***0.16 $\pm$ 0.003	## 0.18 $\pm$0.04

*p value*s

* $P<0.0001$ ** $P<0.001$ @ $P<0.0001$ # $P<0.0001$ $P=0.0062$
© $P= 0.0202$ ® $P= 0.0031$ *** $P= 0.0119$ $P = 0.0081$ ## $P= 0.0239$

Table (4): Prevalence of serum GPC3 levels in aging pancreatic cancer patients

Parameter	GPC3 *(OD)*
Pancreatic	0.31 $\pm$ 0.10
* > 55 years	0.319 $\pm$ 0.10
**< 55 years	0.304 $\pm$ 0.10

* NS   ** $P = 0.6009$
- When comparing the pancreatic cancer patients aging > 55 years with pancreatic aging > 55 years $P= 0.6006$ considered not significant (*NS*)
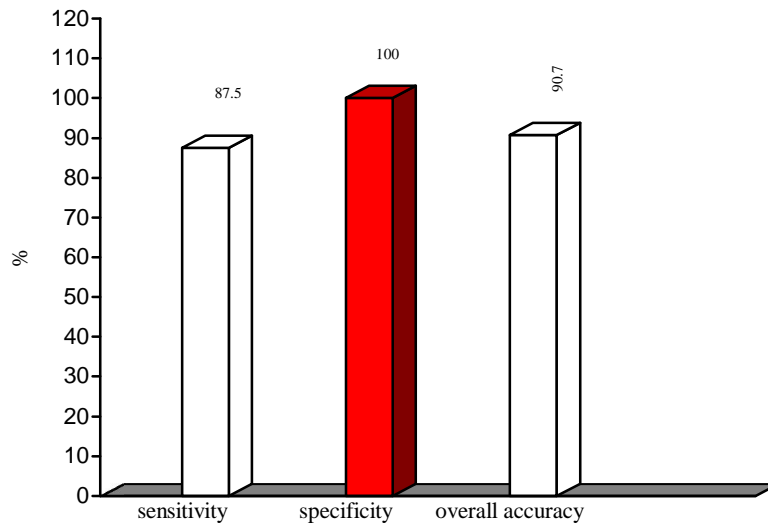

Fig (1): Sensitivity, specificity and overall accuracy of ELISA for detecting GPC3 in pancreatic patients compared to healthy individuals
Sensitivity = True +Ve / True +Ve + false –Ve
Specificity = True –Ve / false +Ve + true –Ve
Overall accuracy = True +Ve + true –Ve / true +Ve + false +Ve + true –Ve + false -Ve

4. Discussion

In the present study, ELISA technique based on monoclonal antibody was used for serum GPC3 quantitation. Previous studies quantitated serum GPC3 in ovarian cancer (Lin et al., 2005), breast cancer (Murthy et al., 2006) and melanoma (Nakatsura and Nishimura, 2006) using ELISA as well. The present results showed that there was a significant elevation in serum GPC3 level in pancreatic cancer patients with respect to the healthy

individuals. GPC3 was detected in serum of 35 out of 40 pancreatic cancer patients with a sensitivity of 87.5% compared to healthy individuals, and with specificity of 100 %, while the overall accuracy of the test was 90.7%. Comparatively, a large number of potential tumor markers have been previously evaluated in pancreatic cancer, but none has been satisfactory either sensitive or specific in detecting pancreatic cancer (Shahi et al., 2002, Sevinc et al., 2003 & Hayashi et al., 2004).

As an endoglycosidase degrading heparane – sulfate proteoglycans (HSPGs), heparanase-1 (HPR1) was over expressed in a variety of malignancies (Parish et al., 2001, Vlodavsky et al., 2002 & Vlodavsky et al., 2003), primary pancreatic adenocarcinomas (Kim et al., 2002 & Rohloff et al., 2002) and pancreatic cancer (Kolipanase et al., 2001). It is suggested that the modification of cell surface HS levels by HPR1 can affect the proliferation of pancreatic cancer cells in response to endogenous or exogenous fibroblast growth factor (FGF). Also, HPR1 expressed in pancreatic adenocarcinomas can suppress the proliferation of pancreatic tumor cell in response to the growth factors that require HSPGs as their co-receptors (Xiulong et al (2007).

The present results showed also that there was a significant difference in serum GPC3 level between pancreatic and stomach cancer patients from one side and between pancreatic and esophageal cancers patients from the other. These findings may encourage the use of the estimation of serum GPC3 level as a prognostic marker for pancreatic cancer. Zhu et al. (2002) found that GPC3 may be involved in the growth control of normal esophageal and gastric epithelial cells. They also suggested that GPC3 may play a tumor suppressor role in gastric but not in esophageal cancer. Furthermore, Tetsuya et al. (2005) found that serum GPC3 was detected in 40% of melanoma patients compared to 30% if conventional markers were used, irrespective of clinical stages.

Since the present results revealed that there were no significant differences in serum levels of AFP levels between pancreatic cancer patients and controls, so, the estimation of serum GPC3 is preferred as a more sensitive tumor marker for pancreatic cancer patients. This suggestion agrees well with the results of Jiang et al. (2004) who found that serum AFP was the least sensitive among other tumor markers e.g. CA 19-9, CA 242, and CA-50 in the diagnosis and follow up of operated cases of pancreatic cancer. Also previously, Radhi and Lukie (1998) found that the pancreatic tumor cells expressing many tumor markers as p53 and CEA were negative for AFP.

The level of serum GPC3 in male pancreatic cancer patients was nearly the same as that in female patients. Furthermore, the level of serum GPC3 in pancreatic cancer patients with age less than 55 years was nearly similar to that of patients with age more than 55 years meaning that serum GPC3 levels are not age-related

On the light of the present results, one can say that GPC3 is over expressed in pancreatic cancer patients with subsequent release and accumulation in the blood circulation. This accumulation enables the use of ELISA, as a noninvasive technique optimized based on specific monoclonal antibody (GPC3 - mAb) to estimate GPC3 in serum of pancreatic cancer patients as a diagnostic test. The absolute specificity (100%) and the relatively high sensitivity (87.5%) of the test prompted us to suggest the use of the estimation of serum GPC3 as a diagnostic and prognostic marker preferred than AFP in pancreatic cancer patients.

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