RESEARCH ARTICLE

Studying the Impact of Golgi Protein 73 Serving as a Candidate Biomarker in Early Diagnosis for Hepatocellular Carcinoma among Saudi Patients

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Abstract

Background: Due to the prevalence of Hepatocellular carcinoma (HCC) in Saudi Arabia, using new markers to give best diagnostic performance than alpha-feto protein (AFP) are important in early diagnosis. The aim of this work was to compare the significance between serum and mRNA Golgi glypican73 (GP-73) as newly identified diagnostic and prognostic markers for HCC among Saudi patients. **Materials and Methods:** A total of 300 subjects were divided into: 250 blood samples where 145 samples from HCC, 105 samples from chronic liver cirrhosis (CLC) and 50 normal controls were investigated for serum GP73 (sGP73) by ELISA. GP-73 mRNA from peripheral blood mononuclear cells was amplified by RT-PCR. The sensitivity and specificity of both techniques was compared. **Results:** Serum Golgi glypican 73 was significantly higher in HCC group compared to cirrhotic and normal controls (p<0.001). Sensitivity and specificity were 95% for sGP-73, 100% and 90% for Golgi glypican 73 mRNA. The combination of sensitivity between AFP and sGP73 was 80% and 95% respectively. **Conclusion:** Both serum Golgi glypican-73 and GP-73Mrna are good diagnostic biomarkers for early detection of HCC in Saudi patients. RT-PCR is more accurate and sensitive (100%) than ELISA (95%) in detecting Golgi glypican 73.

Keywords: Golgi glypican73- Sandwich ELISA- RT-PCR- early surveillance- HCC- Saudi Arabia

Introduction

According to Saudi Cancer Registry, 2010, Hepatocellular Carcinoma (HCC) was detected in 4.8% of all newly detected cases of all types of cancer in Saudi patients. It ranked fourth in males and eighth in females with a male to female ratio of 2.1 : 1 and it accounts for 83% and 80% of all liver cancers in males and females, respectively (Saudi Cancer Registry, 2010).

In fact, the majority of HCC are associated with a background of chronic liver diseases. Therefore, development of HCC is hypothesized to involve multiple genetic changes over a long period of time. Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) contributes significantly to the causal agent of HCC. The great majority (nearly 80%) of HCC cases worldwide is associated with chronic infections with these two viruses (APA Liver consensus recommendations on HCC 2014). But in Saudi Arabia, Hepatitis C is also common with a prevalence rate of 1% to 3% of the population which

further increases the risk of HCC (Madani, 2007; KSA Ministry of Health Statistics 2009).

The highly costs of a diagnostic program of liver malignant is considered the main problem in continuous follow-up. About 30% of liver cancer cases are without elevation in serum alpha-fetoprotein (AFP) levels. However, in other cases AFP levels were characterized with sensitivity and specificity when tumor size is more than 3 to 5 cm in hepatocellular carcinoma (HCC) (Abdo et al., 2012; Flores and Marrero 2014). Therefore, the identification of new biomarkers with high sensitivity and specificity than AFP could be helpful in early diagnosis of Hepatocellular carcinoma (HCC) (Gish 2014; Shaker et al., 2017).

Golgi glypican 73 (GP73) also named Golgi phosphoprotein 2, which was introduced as diagnostic and prognostic marker to identify HCC (Kladney et al., 2000). GP73 is a resident Golgi-specific membrane expressed by biliary epithelial cells and is enhanced in HCC cells (Mao et al., 2010). It has been found and is expressed in

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HBV, HCV infection, alcoholic liver disease, also at high concentration in HCC patients (Anand et al., 2015).

This study advocates evaluating and comparing the sensitivity and specificity of serum and mRNA Golgi glypican 73 in diagnostic HCC patients from cirrhotic patients and normal controls and compared them with AFP. Also, it compares the sensitivity of ELISA and RT-PCR.

Materials and Methods

Exclusion and Inclusion criteria for all patients

Samples were collected from patients with a medical record and continuous follow-up to (ultra sound (US), MRI or CT) the level of liver cirrhosis and liver cancer and exclusion of cases that were met or did not have a medical record. Tumor staging was determined using the United Network of Organ Sharing–modified tumor-node-metastasis staging system for HCC.

All studied subjects were collected from Pathology Department at King Faisal Specialist Hospital and Research Center (KFSHRC, Riyadh and Jeddah), Pathology Laboratory at Saudi German Hospitals (Riyadh, Jeddah, and Medina), Ministry of National Guard Hospitals, Princess Nourah Oncology Center, Jeddah, Security Forces Hospital Riyadh, Armed Forces Hospitals (Riyadh, Western, South and Northern), Public and Privet Hospitals in different regions in Saudi Arabia from July 2014 to May 2017.

Subjects with HCC on biopsy, a new hepatic defect, showed vascular enhancement on one imaging modality (US, MRI, or CT) with AFP >1,000 ng/mL. For the cirrhosis group, patients with hepatitis C and hepatitis B and biopsy-proven cirrhosis were enrolled. All controls healthy group were screened for hepatitis viruses before enrollment.

In the current study, tests were conducted on 300 blood samples, 250 blood samples of Hepatocellular carcinoma (HCC), chronic liver cirrhosis (CLC) and 50 blood samples of healthy control normal group. A total blood samples were 250; 185 (74%) males and 65 (26%) females with an age range from 23 to 78 years. The control group included 50 healthy subjects (blood donors 16 males and 4 females) with an age range between 22 and 60 years. Demographic and clinical information were obtained and a 20-ml blood sample was collected from each subject in a serum separator tube and spun within 2 h, and Centrifuged serum was stored at −20°C for GP-73 protein and AFP assay.

The study was conducted after approval from the ethics committee of Institute Review Board (IRB), King Khalid University Hospital, King Saud university, College of Medicine, (14/4180/IRB). All written approvals were taken from patients before starting the study.

Detection of tumor markers

Serum Golig glypican -73 (GP73) was investigated by using a commercially available ELISA kit provided by LifeSpan (LifeSpan BioSciences, USA). A monoclonal antibody specific for GP73 is pre-coted onto a microplate. Five standard sample wells and one blank control well were assigned to each sample. The sample dilution of 100 μl and test specimen of 15 μl were pipetted into each well, supplemented with standard samples of 0 ng, 20 ng, 50 ng, 120 ng and 250 ng (15 μl each). The microplates were sealed with sealing tapes and incubated at 37°C for 60 min. The plates were sealed with sealing tapes again and incubated at 37°C for 30 min. The plates were thoroughly rinsed for five times again and each well was supplemented with color reagents A and B (50 μl each) for the color reaction at 37°C for 20 min. Termination solution of 50 μl was added into each well and the wavelength of enzyme labeling instrument was set at 450 nm . The sample GP73 concentration was retrieved from the curve. The normal range of serum GP73 was 0-55 ng/mL and the malignancy was indicated in the case of GP73 >100 ng/mL (Shi et al. 2011).

AFP was detected by using the Cobas601 electrochemiluminescence immunoassay analyzer. It indicated positive in the case of serum AFP > 7 μg/L.

Detection of Golgi glypican- 73 (GP73) mRNA by RT-PCR

Two ml of whole blood were placed in anticoagulant blood test tube (EDTA) for RNA extraction from peripheral mononuclear blood cells (PMBs). RNA extraction from PMBs was completed within 6 h after blood drawn. Extracted RNA was supplemented with ethanol of 2.5× volume and stored at -80°C for the assay of GP73 mRNA (InviTrap Spin Blood RNA Mini Kit). The concentration of total RNA was determined with UV spectrophotometry and 500 ng of total RNA was sampled for the reverse transcription polymerase chain reaction (RT-PCR). For the reverse transcriptase synthesis of cDNA, the reverse transcription reaction system (20 μl) consisted of 5-3 PrimeScript™ buffer (4 μl), Prime- Script™ RT Enzyme Mix I (1 μl), Oligo dT Primer (50 μM, 1 μl) and Random 6 mers (100 μM, 1 μl). RNase-free distilled water (dH2O) was supplemented to reach the total system volume at 20 μl. The reverse transcription was performed at 37°C for 20 min and 85°C for 5 sec.

The GP73 and internal control β-actin quantitative PCR system consisted of SYBR Premix Ex Taq™ (10.0 μl), PCR forward primer (10 μM, 1.6 μl), PCR reverse primer (10 μM, 1.6 μl), DNA template (2.0 μl) and sterile distilled water (4.8 μl), respectively. The RT-PCR was performed as following. Denaturation at 95°C for 10 sec, then 40 cycles of 95°C for 5 sec, 64°C for 30 sec and 72°C for 10sec. The upstream primer sequence of GP73 was 5′-CAGCGCTGATTTTGAGATG-3′ and its downstream primer sequence was 5′-ATGATCCGTGTCTGGAGGTC-3′, with the target gene size at 156 bp. The upstream primer sequence of β-actin was 5′-CGTACCACTGGCATCGTGAT-3′ and its downstream primer sequence was 5′-GTGTTGGCGT-ACAGGTCTTTG-3′, with the target gene size at 452 bp.

Statistical analysis

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Qualitative data were expressed as frequencies and percentages. For quantitative data, a comparison between two groups was done using Mann-Whitney test (non-parametric t-test). A comparison between three groups was done using Kruskal-Wallis test

(non-parametric ANOVA) then post-Hoc "Schefe test" on rank of variables was used for pair-wise comparison. Numerical data were expressed as medians and ranges. Spearman-rho method was used to test the correlation between numerical variables. The Receiver Operating Characteristic (ROC) curve was used for prediction of cut off values. The p-value <0.05 was considered significant. Sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) were calculated for the different markers used.

Results

The demographic features of the included population were as follows: the total population in this study was 300 samples (250 blood samples of (Hepatocellular carcinoma (HCC) and chronic liver cirrhosis (CLC)) and 50 blood samples of normal control). A total blood (serum) samples were 250; 185 (74%) males and 65 (26%) females with an age range from 23 to 78 years. The control group included 50 healthy subjects (blood donors 16 males and 4 females) with an age range between 22 and 60 years.

Two hundred fifty blood samples included 145 HCC (male (95%) 138/145; female (5%) 7/145) with hepatitis viruses were 105 (72%) patients with HCV-positive; 20 (17%) were HBV infection and 20 HCC patients were negative for both viral hepatitis markers. Also, 105 CLC (CLD led to LC without HCC) were 67% males (70/105), 33% females (30/105). Also, 75 (71%) with hepatitis viruses (37/75 (49%) with HCV-positive, 27/75 (36%) with HBV-positive, 11/75 (15%) cases co-infected with
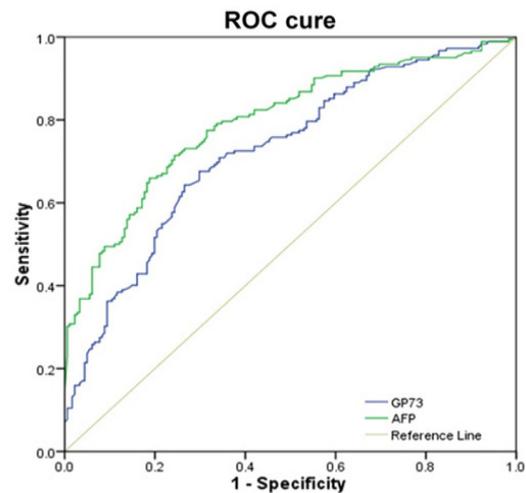

Figure 1. The Roc Curve for Golgi Glypican-73 and AFP

both hepatitis viruses] and 30/105 (29%) patient were negative for both HCV, HBV hepatitis viruses included 10/30 (33%) (Table 1).

Serum Golgi glypican-73 (GP-73) and Alph-feto protein (AFP) show significantly different results in Hepatocellular carcinoma (HCC) compared to cirrhotic (CLC) and healthy normal control group (p<0.001).

Detection of sGolgi glypican -73 by ELISA showed a high positivity rate in HCC 95% (138/145) patients. Compared to CLC, it was 12.4% (13/105) and it was negative in the normal group (p<0.001). Golgi glypican -73mRNA detected by RT-PCR was positive in 100% of cases in HCC group (Figure 2) and it was 26.6% (28/105) positivity in CLC group and was not detected in healthy control group (p<0.001). On the other hand, AFP

Table 1 .The Demographic Feature of the Hepatocellular Carcinoma (HCC) and Chronic Liver Cirrhosis (CLC)

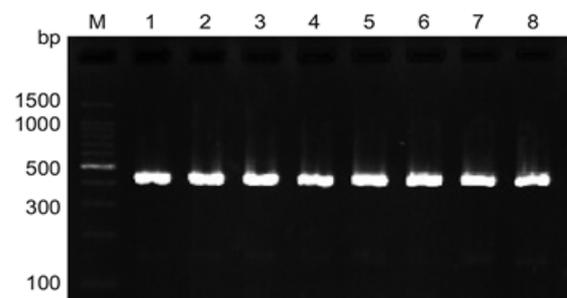| Characteristics	Patients with HCC (n 145)	Patients without HCC (CLC) (n = 105)
Gender (%)	138/145 (95%)	M 70/105 (67%)
Male/Female	7/145 (5%)	F 30/105 (33%)
Hepatitis virus (%)		
HBV	20/125 (16%)	27/75 (36%)
HCV	105/125(84%)	37/75 (49%)
HBV+HCV	N/A	11/75 (15%)
Chronic Hepatitis/LC (%)	125/145 (86%)	75/105 (71%)
Chronic non Hepatitis /L C (%)	20/145 (14%)	30/105 (29%)
Tumor size		
≤ 2 cm	105/145	N/A
> 2 cm and ≤ 3 cm	40/145	N/A

Figure 2. RT-PCR of Golgi Glypican-73 (GP73) Gene Expression in Peripheral Blood Samples. (Lane M, show 100bp of molecular Wight marker, Lane 1,4 Whole blood from HCC, Lane 5, 6 whole blood CLC and Lane 7, 8 control group).

Table 2. Diagnostic Performance of sGP-73, GP-73mRNA Compared with Alpha-Fetoprotein

	s Golgi glypican-73 (cutoff 4.9 ng/ml)	sAFP (cutoff 40.5 ng/ml)	Golgi glypican-73mRNA
Sensitivity% (95% CI)*	95 (85.5-98.7)	80 (70.1-90.7)	100 (91.0-100.0)
Specificity% (95% CI)	95 (85.2-98.7)	80 (70.9-90.5)	90 (68.0-98.0)
Positive Predictive value % (95% CI)	97.5 (88.5-99.7)	91 (80.9-94.9)	95 (83.0-99.0)
Negative Predictive value % (95% CI)	90.5 (79.4-96.2)	70.8 (57.5-81.5)	100 (81.0-100.0)
Diagnostic Accuracy % (95% CI)	95	80.3	96

measurement revealed 80% positivity in HCC group, 25% positivity in the cirrhosis group, and was negative in the normal control group (p<0.001).

Median levels of sGolgi glypican -73 were 125, 62, 0.89 ng/ml and AFP were 147.5, 15, 3.2 ng/ml in the HCC group, cirrhosis group, and normal control group, respectively.

One hundred five out of 145 of the HCC patients (72.4%) were HCV positive and 40/145 (27.6%) were HBV positive. All hepatitis positive HCC patients were cirrhotic.

In HCC patients, the highest results of sGolgi glypican-73 were detected in HBV and HCV positive compared to HBV and HCV negative patients. It was 96% (120/125), 70% (14/20) in positive and negative hepatitis virus HCC patients respectively. As for AFP, the highest results were detected in HBV and HCV positive patients (Table 2). In CLC patients, sGolgi glypican-73 by ELISA were detected in 14.7 % (11/75 in positive HBV and HCV) and 6.6% (2/30 in negative HBV, HCV) and compared to Golgi glypican -73mRNA detected by RT-PCR was positive in 33% (23/75), 16.6% (5/30) in +ve/-ve HBV, HCV respectively.

As shown in Figure 1, the area under curve of GP73 was 0.896 ± 0.027 and its 95% confidence interval was 0.833 to 0.939. GP73 could be used for the diagnosis of early surveillance of HCC. The area under curve of AFP was 0.818 ± 0.034 and its 95% confidence interval was 0.741 to 0.875. Thus, the diagnostic value of GP73 was higher than that of AFP.

As shown in Figure 2, the band of β-actin was 452 bp and that of 156 bp was the target gene of GP73. The expression of the GP73 gene remained constant in normal control while the band density of the target gene GP73 was higher in HCC specimens than in CLC specimens, so expression of GP73 mRNA was significantly up-regulated in HCC specimens than in normal control group.

As for the diagnostic performance of two biomarkers, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy for sGolgi glypican-73 were (95%,95%, 97.5%, 90.5 and 95%) respectively at cut off 4.9 ng/ml and for AFP were (80%, 80%, 91%, 70.8 and 80.3%) respectively at cut off 40.5 ng/ml. For Golgi glypican73 mRNA, they were (100 %, 90%, 95%, 100% and 96%) respectively (Table 2).

Discussion

In fact, the surveillance programs of HCC has evolved significantly over the past few decades (Page et al., 2014). Unfortunately, the most of it depends on alpha fetoprotein (AFP), even in combination with abdominal ultrasound (Mustafa et al., 2013). However, some reports recorded that the sensitivity for the detection of HCC ranges between 25%-60%, and its specificity is low and AFP can also be detected in patients with cirrhosis (11%-47%) and chronic hepatitis without cirrhosis (15%-58%) (Abdulaziz and AL-Salloom, 2016). In addition, 30% HCC patients present with normal AFP. Thus, they needed for new tumor markers that can differentiate between HCC and benign hepatic lesions and this is very important for early

surveillance and specific diagnosis of HCC (Salim et al., 2009; Shaker et al., 2017).

This study was focused on Golgi protein-73 (GP73) as a new biomarker, which is a 73-kD resident Golgi membrane protein of unknown function. GP73 is a type II trans membrane protein with a single, N-terminal trans membrane domain and an extensive, C-terminal coiled-coil domain located on the luminal surface of the Golgi apparatus (Marrero and Romano 2005; Comunale et al., 2006).

It was recently reported that GP73 was detected in biliary epithelial cells in patients with acute hepatitis, cirrhosis and HCC (Marrero and Romano 2005). GP73 values are higher in early HCC patients than in cirrhotic patients; GP73 is considered a possible biomarker for screening the diagnosis of early HCC (Comunale et al., 2006).

In the HCC group, GP-73 mRNA was the most sensitive among the studied markers for diagnosing HCC among Saudi patients with 100% positivity rate, followed by sGP73 (95%), then AFP (80%). Also, in cirrhosis group, GP-73 mRNA showed the highest positivity (49.6%), followed by AFP which was (25%) then sGP-73 (20.7%). The three markers were negative in the normal control group.

A comparison of sGP-73 and AFP between the three studied groups showed a significant difference between HCC and both liver cirrhosis and normal groups. This is in accordance with other researchers (Marrero and Romano 2005; Comunale et al., 2006; Shan et al., 2013; Zhang et al., 2015).Serum levels of GP-73 and AFP levels increased in the HCC patients and were negative in other benign liver cases (Yamaoto et al., 2010). The absence of serum GP-73 in healthy individuals was also reported by some previous studies (Mao et al., 2010; Zhou et al., 2012).

As regards the diagnostic performance, Sensitivities of GP-73 mRNA, sGP73 and AFP were (100%, 95% and 80%). Specificities were (90%, 95% and 80%) respectively. GP-73 mRNA is more sensitive but low specific than sGP73 and both are highly sensitive and specific for diagnosing HCC and differentiating HCC from benign liver conditions and normal controls than does AFP.

Our findings demonstrated that serum GP-73 in HCC group significantly differed from those in cirrhosis, hepatitis and normal control group, indicating that in Saudi patients, GP-73 was significantly elevated in HCC serum as a good biomarker for early surveillance of HCC. That was because of the release of GP-73 protein by hepatoma cells into the circulation in first stage HCC patients, resulting in the significantly elevated sGP73 in HCC. These results indicated that the hepatitis cirrhotic patients also had GP-73 elevated, that was consistent with the study's results of Kladney et al., (2002), Rieneret al., (2009), Hu et al., (2010) and Anand et al., (2015) indicating that GP-73 was highly up-regulated in both acute and chronic hepatic disorders in Saudi patients.

Several studies have shown that Golgi glypican 73 is superior to AFP in early detection of HCC, being highly sensitive and specific (Gu et al., 2009; Zhou et al., 2012; Shan et al., 2013). The comparison of ROC curves for

Gp73 and AFP displayed that GP-73 was more accurate than AFP in the diagnosis of HCC. The sensitivity of sGP-73 was 95% and AFP was 80% in HCC consistent with the findings of Block et al., (2005), Gu et al., (2009) and Hu et al. (2010).

As regards GP-73 expression at mRNA level by RT-PCR, our results revealed that GP-73mRNA was detected in all cases of HCC (100%), in cirrhosis patients was (49.6%) and was not detected in any of the normal healthy controls. Our results were in agreement with Comunale et al., (2006); Riener et al., (2009); Mao et al., (2010); Shi et al., (2011); Shan et al., (2013) these studies reported similar results concluding that GP-73mRNA is significantly up-regulated in HCC compared to normal and benign liver samples, and hence GP-73 mRNA could serve as a molecular tumor biomarker for early detection of HCC.

Regarding the one hundred five cirrhotic patients, included in the present study, twenty eight of them were positive for GP-73mRNA. These cases were followed up for 12 months. The nineteen who were positive hepatitis viruses also have mildly elevated sGP-73 and AFP levels has been diagnosed as HCC after 6 months of follow up. These results could support that GP-73 can be used for screening and early detection of HCC among cirrhotic patients. This was in accordance with Comunale et al., (2006), who demonstrate that during the follow up of their patients with liver cirrhosis having detectable sGP-73 levels, HCC developed within 6 months among a considerable number of patients with a significant change of serum AFP levels.

The lack of correlation between Gp-73 and positivity of HBV and HCV infection in this study proves the high specificity of GP-73 in HCC versus non HCC hepatitis cases, as positivity of HBV or HCV infection will not give false positive results especially in Saudi patients, where there is a continuous prevalence of hepatitis viral infection. Kladney et al., (2000) reported that GP-73 was present in the serum of HCC patients, but was undetectable in all patients with hepatitis as well as healthy individuals. Maitra et al., (2004) proposed that GP-73 was highly expressed in both acute and chronic hepatic patients, indicating that such protein was less helpful for the etiological diagnosis of hepatic disorders but could be used as their serum biomarker. On the other hand, Morota et al., (2011) demonstrated that Gp-73 evaluated in the serum of patients with chronic hepatitis, cirrhosis, and hepatocellular carcinoma.

Comparing the sensitivity of two techniques used, GP-73by RT-PCR proved to be more sensitive (100%) than ELISA (95%).

Trans membrane protein of Golgi apparatus does not seem to play a critical role in tumor progression even not expressed by hepatocytes, the viral infection induced the overall, up regulation expression of GP-73 in hepatocytes HCC, this strongly suggests that GP-73 is a good molecular biomarker for early surveillance of HCC. Estimation of GP-73 diagnostics and immunotherapeutic target may help in prevention and treatment of liver cancer (Kladney et al., 2000; Mao et al., 2010; Shan et al., 2013; Anand et al., 2015).

In conclusion, GP-73 is a highly sensitive and specificity diagnostic biomarker for HCC which can be used in early detection of HCC and in screening and follow up of patients with cirrhosis among Saudi population. There is no impact for the presence of hepatitis viruses on the diagnostic accuracy of Golgi glypican73 in diagnosing HCC among Saudi patients. Measuring GP-73mRNA by RT-PCR proved to be more sensitive (100%) than GP-73 by ELISA (95%), and it is more suitable for follow up of cirrhotic patients. Further studies with larger sample sizes and longer follow up of HCC and chronic liver cirrhosis (CLC) patients are needed to estimate and clarify the role of Golgi glypican73 in early diagnosis of HCC.

Acknowledgments

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