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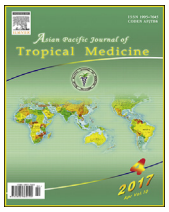

Original research http://dx.doi.org/10.1016/j.apjtm.2017.03.016

Combination of serum tumor markers dickkopf-1, DCP and AFP for the diagnosis of primary hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 15 Nov 2016
Received in revised form 5 Mar 2017
Accepted 16 Mar 2017
Available online 6 Apr 2017

Keywords:
Tumor markers
Diagnosis
Primary hepatocellular carcinoma

ABSTRACT

Objective: To evaluate the detection accuracy of the biomarkers dickkopf-1, DCP and AFP as a serum biomarker panel by comparing the sensitivity of the panel with those of the individual biomarkers.

Methods: The study was composed of three groups, one with HCC patients, one with non-HCC liver diseases and one with healthy controls. Serum AFP was measured using a chemiluminescence assay and serum dickkopf-1 and DCP were measured with ELISA. The sensitivity and specificity of the biomarkers were analyzed as single parameters and as a serum panel.

Results: The HCC group showed higher levels of dickkopf-1, DCP and AFP than the other two groups ($P < 0.05$). Dickkopf-1 showed better sensitivity (73.26% *vs.* 58.13%, $P < 0.05$) and better specificity (44.0% *vs.* 29.0%, $P < 0.05$) than AFP. DCP also had better sensitivity (74.42% *vs.* 58.13%, $P < 0.05$) than AFP, but their specificity was similar (30.00% *vs.* 29.00%, $P > 0.05$). The combination of the biomarkers as a serum panel produced much better sensitivity (93.02%) and specificity (78.00%) than each of the markers individually ($P < 0.05$).

Conclusion: The combination of AFP, DCP and dickkopf-1 as a biomarker panel can significantly improve the detection power with much higher sensitivity and specificity for HCC than any of the biomarkers alone. The tests are convenient and inexpensive, and may serve as a valuable addition to current options for the diagnosis of HCC.

1. Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common types of malignant tumors in the world, with more than three quarters of a million new cases diagnosed annually [1]. In certain developing countries, notably the sub-Sahara region and Southeast Asia, it is the most prevalent cancer, with an early age of onset and a short survival time [2]. More than half of new HCC cases occur in China, as a result of high childhood infection rates of hepatitis B [3]. Despite the implementation of a nationwide hepatitis B vaccination program for children for nearly two decades, it may take many years to achieve an appreciable reduction in HCC incidence and mortality [4]. The prognosis of HCC is generally poor and critically dependent on the extent of liver cirrhosis and tumor staging. Therefore, early detection is the key to favorable treatment outcomes.

Most HCC cases are asymptomatic in the initial stages and make early diagnosis difficult. Although advanced imaging technologies such CT and MRI have become increasingly available and greatly facilitated detection and characterization of small lesions of the liver, serum α-fetoprotein (AFP) and ultrasound examination remain the most common methods for the diagnosis of HCC, especially in high-risk third world areas, and are frequently unable to differentiate between early liver cancer and other types of small liver lesions [5]. Therefore, convenient and cost-effective detection methods for HCC are highly desirable. Advances in molecular biotechnology have identified a

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Peer review under responsibility of Hainan Medical University.

number of biomarkers that are closely associated with tumor development and progression. Of those, dickkopf-1, an inhibitor of the Wnt signaling pathway, is implicated in several types of cancer and is thought to be a promising biomarker for liver cancer [6,7]. Likewise, des-γ carboxyprothrombin (DCP), an abnormal form of prothrombin, has been used as a marker for hepatocellular carcinoma for more than three decades [8,9]. However, dickkopf-1 or DCP alone has shown less than satisfactory sensitivity and specificity for the detection of HCC [10]. In this study, we explored the possibility of combining dickkopf-1, DCP and AFP as a serum biomarker panel to improve the diagnosis of HCC.

2. Materials and methods

2.1. Patient selection

This study was performed at People's Hospital of Lingao County and the research protocol was approved by the Ethics Committee of the hospital. A written informed consent was obtained from all participants before the commencement of the study. Participants were recruited during the period of January 2015 to May 2016 and were assigned to one of three groups, based on their eligibility. The HCC group contained 86 microscopically confirmed HCC patients, 52 were male and 34 were female, aged between 36 and 68 years. The non-HCC liver disease group had 50 patients, 31 were male and 19 were female, aged between 33 and 67 years, of whom 18 were diagnosed with hepatitis B, 16 with liver cirrhosis, 9 with steatosis and 7 with hepatic hematoma. The healthy control group had 50 individuals, 32 of them were male and 18 were female, aged between 30 and 70 years, who had recently undergone comprehensive health screening and met the following enrolment criteria: 1) no liver disorders; 2) no serious abnormalities of other major organs and systems; 3) normal blood routine and liver and kidney function; 4) no abnormal findings on chest and abdominal X ray and ultrasound examination. There is no significant difference in mean age or male and female ratio among the three groups.

2.2. Measurement of dickkopf-1, DCP and AFP

To prevent potential interference with the measurement of the markers by medications, blood samples were collected before the start of drug treatment for patients in both the HCC group and the non-HCC liver disease group. Blood was drawn into serum separator tubes before breakfast. After setting for 30 min, samples were centrifuged at 3000 r/min for 10 min and then stored in a freezer at −80 °C until further use. Serum AFP was measured using the Beckman DxI 800 automated chemiluminescence analyzer (Beckman Coulter, Inc., Brea, CA, USA), with reagents from a Roche diagnostic kit (Roche Diagnostics, Shanghai). Enzyme-linked immunosorbent assays (ELISA) were conducted to measure serum dickkopf-1 and DCP levels. ELISA kits for dickkopf-1 and DCP were purchased from Boster Biotechnologies (Boster Biotechnologies, Wuhan) and microplates were read using the Perlong DMN-9602G reader (Perlong Medical Equipment, Nanjing). The assays were performed with strict adherence to the protocols and instructions specified by the manufacturers. The upper limit of the reference range was 10 ng/mL for AFP, 4 ng/mL for DCP, and 2.0 ng/mL for dickkopf-1. Results exceeding the upper limit were considered positive.

2.3. Statistical analysis

All statistical analyses were conducted using SPSS version 17.0 for Windows (SPSS, Inc., Chicago, IL), and significance was set at 0.05 for all tests. Measurement data were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was performed to detect differences among the three groups, and Fisher least significant difference was used for post hoc tests for comparison of means between any two groups. The χ^2 test was conducted for comparison of nominal data between groups.

3. Results

3.1. Distribution of participants by gender and age among the three groups

The HCC group, the non-HCC liver disease group and the normal group were similar in gender composition, range of age and mean age. ANOVA and χ^2 test revealed no differences among the groups.

3.2. Levels of dickkopf-1, DCP and AFP among the three groups

As can be seen from Table 1, the HCC group showed the highest levels of dickkopf-1, DCP and AFP among the three groups. The differences were statistically significant between the HCC group and the non-HCC group ($P < 0.05$) and between the HCC group and the normal control group ($P < 0.05$). Levels of the three markers were all slightly higher in the non-HCC group than in the normal control group, but the differences have no statistical significance for any of the markers.

3.3. Comparison of sensitivity and specificity of dickkopf-1 and AFP for the diagnosis of HCC

In this study, the sensitivity of a marker was defined as the percentage of HCC patients who were seropositive for that marker, while the specificity of a marker was defined as the percentage of non-HCC liver disease patients and normal controls who were seronegative for that marker. The sensitivity of dickkopf-1 was 73.26% (63/86), higher than that of AFP, which was 58.13% (50/86), and the difference was statistically significant ($P = 0.011$, $\chi^2 = 6.261$). Similarly, dickkopf-1 showed higher specificity 44.00% (44/100) than AFP, whose specificity was 29% (29/100), and the difference was significant ($P = 0.04$, $\chi^2 = 4.787$) (Table 2).

Table 1
Levels of dickkopf-1, DCP and AFP among the three groups (ng/mL).

Group	*n*	dickkopf-1	DCP	AFP
HCC	86	3.85 ± 1.34[a,b]	8.15 ± 3.31[a,b]	356.72 ± 42.57[a,b]
Non-HCC	50	1.82 ± 0.45	2.65 ± 0.68	43.46 ± 8.05
Control	50	1.43 ± 0.25	2.13 ± 0.67	6.85 ± 3.42
F		123.42	163.11	374.21
P		0.000	0.000	0.000

[a] Significantly different from the non-HCC group, $P < 0.05$. [b] Significantly different from the normal control group, $P < 0.05$.

Table 2
The power of detection of the combination of dickkopf-1, DCP and AFP vs. a single marker (%).

Marker (s)	Sensitivity	Specificity
dickkopf-1	73.26 (63/86)	44.00 (44/100)
DCP	74.42 (64/86)	30.00 (30/100)
AFP	58.13 (50/86)	29.00 (29/100)
Combination of dickkopf-1, DCP and AFP	93.02 (80/86)	78.00 (78/100)

3.4. Comparison of sensitivity and specificity of DCP and AFP for the diagnosis of HCC

Unlike dickkopf-1, DCP, as a biomarker for HCC, was superior to AFP only in sensitivity, when assessed by the two parameters. Specifically, the sensitivity of DCP was 74.42% (64/86), whereas the sensitivity of AFP was 58.13% (50/86), and the difference was statistically significant (P = 0.03, χ^2 = 8.450). The specificity of DCP was 30% (30/100), lower than that of dickkopf-1 (44%), it was not significant different from that of AFP (P = 0.728, χ^2 = 0.121) (Table 2).

3.5. The power of detection of the combination of dickkopf-1, DCP and AFP vs. a single marker

As described above, when used alone, dickkopf-1, DCP and AFP showed varying levels of sensitivity and specificity. DCP and dickkopf-1 had similar sensitivity, but the latter had better specificity. AFP had the lowest sensitivity and specificity of the three markers. In order to improve the power of detection, we tried to use the three markers as a serum panel. As Table 2 shows, the combination produced far better sensitivity and specificity than each of the markers alone. When all three markers were seropositive, the sensitivity was 93.02%, compared with that of each of the markers (χ^2 = 11.90, 10.92 and 28.35, respectively, $P < 0.05$), and the specificity, at 78.00%, represented an even more pronounced improvement over that of a single marker (χ^2 = 29.32, 12.50 and 11.49, respectively, $P < 0.05$).

4. Discussion

The present study has demonstrated the superiority of serum dickkopf-1, DCP and AFP as a test panel to individual biomarkers for the diagnosis HCC. In addition to clinical manifestations, ultrasonic imaging and other methods, clinicians are heavily dependent on serum markers, such as AFP, for the detection of HCC, especially in areas where advanced diagnostic technologies are not available [5]. However, AFP has shown insufficient sensitivity and specificity [10]. Although the Asian Pacific Association for the Study of the Liver still includes AFP for the diagnosis of HCC, the suggested cut-off level is 200 ng/mL, far higher than the upper limit of the normal range [11]. In its latest guidelines, the American Association for the Study of Liver Diseases no longer recommends AFP as part of the evaluation measures [12]. In attempts to find other diagnostic markers for HCC, investigators have examined other biological molecules. In a study that combined AFP with dickkopf-1 and osteopontin (OPN), a sensitivity of 88.76% was reported [13]. Using deep plasma proteome analysis, another study found significantly elevated levels of latent-transforming growth factor β binding-protein 2 (LTBP2) and OPN in HCC patients when compared with chronic liver disease patients and normal controls, and the combination of LTBP2 and OPN was able to identify patients with AFP levels below 20 ng/mL but at high risk of developing HCC [14]. These markers have yet to be adopted in routine laboratory tests.

Since HCC is highly prevalent in many parts of the world, early detection and appropriate management will have an enormous impact on promoting survival time and quality of life. Unfortunately, the cancer is often diagnosed in late stages in a significant portion of HCC patients and treatment options are very limited. For many African and Asian countries, serum AFP and ultrasonography are still the mainstays of diagnosis for HCC, but their value is limited for accurate assessment of small lesions in the liver [15]. As a conventional marker for liver cancer, AFP is frequently undetectable or is only expressed at very low levels when tumors are less than 3 cm in size. Furthermore, elevated levels of AFP are also seen in hepatic cirrhosis, chronic hepatitis and other types of tumors [16]. Several studies have shown that the detection power of AFP for early stage HCC varies considerably and, at high levels, its sensitivity is within the 40%–65% range and specificity within the 76%–96% range [17,18]. Therefore, there is an urgent need to seek new biomarkers with better detection capabilities. Ideally, a biomarker for HCC should be highly sensitive, can differentiate HCC from other types of liver lesions, and is expressed at high levels in early stages. In our study, serum dickkopf-1, DCP and AFP failed to meet the standards as single markers, but their combination greatly improved the detection power for HCC.

As a precursor of prothrombin, DCP is synthesized in the liver and is induced by vitamin K deficiency. Normally, vitamin K-dependent γ-glutamyl carboxylase converts a precursor into prothrombin with vitamin K as a cofactor [19]. In the process, 10 glutamic acid residues become γ-carboxylated residues. When vitamin K is absent or deficient and the activity of γ-glutamyl carboxylase is reduced, fewer than 10 glutamic acid residues become γ-carboxylated, the resulting protein is known as DCP [20]. Malignant liver cells have lower vitamin K levels than normal liver cells [21]. DCP levels are extremely low and are not detected in healthy individuals, but they are elevated in patients with HCC or other liver disorders [10]. The association of DCP with HCC was first reported in a 1984 study, in which 91% of HCC patients were found to be DCP-seropositive and low levels of DCP were also detected in chronic active hepatitis and slightly higher levels in metastatic carcinoma involving the liver. In addition, surgical resection of tumors in some patients lowered the concentration of DCP, which went up with disease recurrence [22]. Subsequent studies have demonstrated that DCP offers better sensitivity as a marker than AFP for HCC, and this is consistent with our findings [23]. Some studies have also reported that DCP can be detected in AFP-seronegative HCC patients, thus offering another advantage over AFP [24].

Dickkopf-1 is an inhibitor of Wnt, a signaling pathway that is evolutionarily well conserved and exists in all species. Wnt participates in embryonic development and tissue regeneration, and its activation leads to uncontrolled cell proliferation and growth, resulting in the development of many types of cancer [25]. The involvement of dickkopf-1 in cancer is complicated and its expression appears to depend on the type of tumor tissue. Increased expression of dickkopf-1 has been found in liver

cancer, lung cancer and esophageal cancer, while decreased expression has been reported in colon cancer and cervical cancer [26]. In HCC patients, dickkopf-1 has been used to assess prognosis and overexpression of dickkopf-1 is often closely related to poor survival outcomes. One study with Chinese HCC patients revealed that those with higher dickkopf-1 levels had both a lower 5-year overall survival rate and a disease-free survival rate, compared with patients with lower levels of dickkopf-1 [27]. Similar to DCP, dickkopf-1 can be detected in a subgroup of patients whose AFP levels are within the normal range [28]. Our data clearly indicate that dickkopf-1 levels in the HCC group were much higher than in the non-HCC group and the normal control group. As a single parameter, dickkopf-1 showed higher sensitivity and specificity than AFP. The results suggest that both DCP and dickkopf-1 can serve as complementary markers for AFP and justify their inclusion in laboratory tests for the diagnosis of HCC.

In conclusion, our study has demonstrated that the combination of AFP, DCP and dickkopf-1 as a biomarker panel can significantly improve the detection power with much higher sensitivity and specificity for HCC than any of the biomarkers alone. The tests are convenient and inexpensive, and will be a valuable addition to current options for the diagnosis of HCC. Future studies should be directed at clarifying several issues. By using larger sample sizes, more accurate estimates for the sensitivity and specificity of the panel will be established. Since levels of the markers are much higher in HCC patients than in non-HCC patients and healthy individuals, different cut-off levels can be tried to further improve the specificity. Finally, patients may be grouped based on staging to assess the value of the panel in the detection of early stage HCC.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was supported by an Applied Research and Development Promotion grant of Hainan Province (No.: ZDXM2014074) and a grant from the Program of Social and Scientific & Technological Development Projects of Hainan Province (No.: SF201422).

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