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EXPERIMENTAL STUDY

Clinical utility of biomarkers of hepatocellular carcinoma

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ABSTRACT
Hepatocellular carcinoma (HCC) is one of the most common forms of cancer significantly affecting the mortality and morbidity rates. The increasing incidence of HCC is a great concern across the globe. The current methods of HCC screening, detection and diagnosis depend mainly on imaging techniques. However, biomarkers represent a relatively easy and noninvasive way to detect and estimate the disease prognosis. New potential biomarkers such as α-fetoprotein (AFP), des-γ-carboxyprothrombin (DCP), α-fetoprotein L3 (AFP-L3), glypican 3 (GCP3), micro-RNA, and Golgi-protein 73 (GP73) are being used more often in the diagnosis and prognosis of HCC. The lack of prudent diagnostic measures makes early detection of HCC nearly impossible. The use of biomarkers to detect cancer has helped to screen for the disease. However, the most commonly used biomarkers for HCC have inadequate performance characteristics. Despite numerous efforts to identify molecules as potential biomarkers, there is no single ideal marker for HCC. In this paper the main biomarkers for the surveillance, diagnosis and prognosis of HCC are reviewed. The advantages and limitations of these biomarkers are summarized, and the future development directions are proposed *(Tab. 1, Ref. 30)*. *Text in PDF www.elis.sk*
KEY WORDS: hepatocellular carcinoma, biomarkers, AFP, DCP, diagnosis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent forms of malignancy and the third leading cause of cancer-related mortality globally (1) with more than 50% of the cases diagnosed specifically in China (2). A survey of the global incidence of liver cancer by the Global Cancer Statistics in 2018 reported that liver cancer was the sixth most prevalently diagnosed cancer on the globe (3). Despite the overwhelming incidence of hepatic cancer in Asia and Africa (3), it was relatively less prevalent in the Western world. Notwithstanding, the past three decades have seen a tremendous rise in HCC incidence, almost doubling in the United Kingdom and tripling in the United States (4). In the United states, mortality from liver-related diseases has been attributed to alcoholic liver disease, nonalcoholic fatty liver disease and hepatitis C (1). There is a relatively high incidence of hepatocellular carcinoma cases in the male population compared to women (5). As at 2008, hepatocellular carcinoma was the 5th prevalent cancer in adult men and 2nd cause of mortality in the male population globally (6). Apart from some other well-known less prevalent cancer forms like angiosarcoma, hemangiosarcoma and hepatoblastoma, liver cancers occur primarily in two main distinct forms, hepatocellular carcinoma and intrahepatic cholangiocarcinoma (ICC). Hepatocellular carcinoma originates from the liver parenchymal cells and accounts for about 80 % of all global cases. It is the most common form of liver cancer cases recorded (5).

Multiple factors contribute to the development of HCC, including alcohol, obesity, and diabetes. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are recognised as leading aetiological agents of HCC worldwide. HBV is considered a major risk factor for HCC across the globe (7).

The diagnosis of HCC at the earlier stages of development is crucial for providing effective curative therapeutic options in liver transplantation, liver resection or microwave ablation. Additionally, it plays a role in prolonging survival through transarterial chemoembolization (TACE) (8). However, at its early stages, HCC is asymptomatic, and the majority of the screening and detection is done and observed at the advance stages. Modern medical technology remains the only reliable way for assessing an early diagnosis of HCC. The currently available clinical procedure includes radiological screening and monitoring for patients with defined risk factors (liver cirrhosis, viral or chronic hepatitis, NAFLD, etc.) but this procedure requires skilled personnel and test equipment that is not readily available (8). Serum biomarkers are critical tools for surveillance and early diagnosis of HCC because they can be collected in a non-invasive manner, and their assessment is objective and reproducible (9). Biomarkers associated with HCC including α-fetoprotein (AFP), des-γ-carboxyprothrombin (DCP) and lectin-bound AFP (AFP-L3%) obtained from body fluids or tissues represent a promising approach to improving the diagnostic accuracy and reducing the limitations and disadvantages of current

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diagnostic procedures. This approach is particularly beneficial for both patients and clinicians when non-invasive technique such as blood or serum assessment is employed (10, 11)

Some improvements have been made over the past decade in the therapeutic approach to both early and advanced cases of HCC development. The guidelines for the diagnosis of HCC issued by the European Association for the Study of the Liver along with the American Association for the Study of Liver Diseases recommend abdominal ultrasound with or without α-fetoprotein (AFP). Current guidelines for HCC surveillance are primarily based on imaging methods (12, 13). However, the reliability of ultrasound depends largely on the experience of operators, and is often affected by liver cirrhosis and fat, as well as by size of hepatic lesions. For patients at high risk for HCC, the surveillance based upon abdominal ultrasound (US) is used as the first-line modality, followed by dynamic computed tomography (CT) or magnetic resonance imaging (MRI) (7). Conversely, no significant advance has been made in the improvement of surveillance and diagnosis by way of clinical HCC biomarkers that might play a noteworthy role in establishing a more accurate prognosis and improving the treatment response (14). In recent times, many serum biomarkers, such as α-fetoprotein (AFP), γ-glutamyl transferase (GGT), and carcino-embryonic antigen (CEA) are used in the assessment of survival outcomes of HCC patients, with AFP widely accepted as a biomarker for HCC prognosis. However, the sensitivity and specificity of these biomarkers is limited, which makes it difficult to accept their clinical utility and reliability (2). Consequently, several other biomarkers have been suggested to complement AFP and increase the accuracy of HCC detection, most notably des-γ-carboxyprothrombin (DCP), lectin-bound AFP (AFP-L3%), osteopontin (OPN), glypican 3 (GPC3) and Golgi protein-73 (GP73). There is however a disparity in the reports of clinical performance of these markers and although the combination of AFP with DCP and AFP-L3% has a moderately enhancing effect on the diagnostic performance compared to their separate use, a comprehensive meta-analysis has shown both DCP and AFP-L3% to be inferior to AFP (10).

In this review we summarized the main HCC biomarkers used in the surveillance and diagnosis of HCC while focusing on their advantages and limitations and potential use as diagnostic, prognostic and therapeutic targets in HCC. Finally, we provide an outlook for future directions.

Biomarkers of HCC

Circulation biomarkers help in the screening, surveillance, prediction of prognosis and monitoring of response to therapy of cancers because they are easily accessible and non-invasive. With the continuous improvement and development of new biomarkers, significant gains are made in the diagnosis and surveillance of HCC(10). A summary of the serum biomarkers of HCC and their advantages and limitations are captured in Table 1.

Alpha-fetoprotein (AFP)

AFP is a globulin with a molecular mass of about 70,000 Daltons. It contains a glycoprotein with 591 amino acids. At the initial stages of fetal life, AFP is produced by the yolk sac. At a more progressed stage, this role is transitioned to the fetal liver. Immediately after birth, a sudden decline in the serum levels of AFP often takes place. The level of AFP becomes elevated in cases of acute liver injury as a response to active regeneration of liver cells. Studies conducted on serum specimens obtained before and after surgical resection of the liver have confirmed that the rapid increase in hepatocytes was correlated with the generation of AFP whilst in context of liver failure due to acetaminophen liver injury, the increase in AFP has been strongly associated with a favourable outcome (7). AFP has been commonly used in clinical practice as a traditional biomarker for HCC surveillance over the past decades. Its significance was initially demonstrated by Abelev et al. in mice and by Tatanirov in patients with liver cancer. Subsequent studies conducted by Force et al. and Song et al. also recognized AFP as a biomarker (7, 9). The sensitivity and specificity of diagnostic biomarkers are always influenced by the chosen cut-off

Tab. 1. Overview of the advantages and limitations of HCC biomarkers.

Biomarker	Advantage	Limitation	Reference
Alpha (α) fetoprotein	Utilized in screening, surveillance, and prognostication	Low sensitivity and specificity	(7,9,13,15)
Des-γ-carboxyprothrombin	Has better performance for the diagnosis of HCC >5cm,	Diseases inducing vitamin K deficiency limits its diagnostic value, false elevation in non-HCC patients	(13,16–18)
Alpha (α) fetoprotein L3	Measurement can be made in patients with AFP levels as low as 2 ng/mL, it is used for an early diagnosis of HCC when the tumour diameter is <2 cm	Requires specialized equipment, it is expensive	(19–22)
Glypican 3	GCP-3 has a higher specificity greater than 95%, can signal detection of HCC even when AFP and DCP are negative	Low sensitivity	(23,24)
Micro RNA	Stable, resistance to RNase activity	Variable annotation with characterization of miRNA	(19,25–28)
Golgi-protein 73	Can distinguish between benign and malignant tumours, useful for diagnosis, surveillance, recurrence and post -operative management	The western blot technique is complex	(18,19,29,30)

value. As the cut-off value is incrementally adjusted, the specificity tends to increase while the sensitivity declines. The previously used AFP value > 400 ng/ml served as a reference for HCC diagnosis. However, this compromised the sensitivity given that around 70 % of patients with HCC had AFP levels < 100 ng/ml. Consequently, most clinicians resorted to the cut-off value of 20 ng/ml as to allow for early detection of HCC with sensitivity and specificity rates of 60 % and 90.6 %, respectively (13). The clinical utility of AFP in screening, surveillance and prognostication of HCC is unquestionable. Liver biopsy can be easily conducted in patients with significant increases in AFP to confirm the diagnosis. It has been shown that AFP negativity was a favourable predictor of liver transplantation eligibility (15) although its validity as a biomarker is undermined by low sensitivity and specificity. While AFP is currently used as a biomarker in clinical settings alongside ultrasound, it lacks the requisite specificity and sensitivity to stand alone as a powerful biomarker.

Des-γ-carboxyprothrombin (DCP)

Des-γ-carboxyprothrombin (DCP) also known as prothrombin induced by vitamin K absence/antagonist-II (PIVKA-II) is produced in HCC as a result of defective decarboxylation of the prothrombin precursor after translation and represents yet another new serological biomarker of HCC (16). DCP is another serum protein that has shown its potential as a biomarker and studies have proven it to be superior to AFP, specifically for larger tumours and those with viral aetiology (17). DCP is known to have a better performance for the diagnosis of HCC > 5 cm. The advancements in technology involved in the detection and measuring of biomarkers have been used to improve the potential of DCP by enhancing the initially limited performance of the conventional DCP assay. The modern DCP assay uses an electrochemiluminescence immunoassay sandwiched with 2 monoclonal antibodies, namely P-11 and P-16 (termed NX-DCP). It provides for the detection of non-specific elevations of DCP due to vitamin K deficiency whilst the conventional DCP assay detects both DCP elevations induced by HCC and those induced by vitamin K deficiency, using the MU-3 antibody (18). Considering that NX-DCP is not elevated in patients with HCC, the patients presenting with HCC have a greater DCP/NX-DCP ratio compared to non-HCC individuals. Thereby, this new DCP/NX-DCP ratio development increases the specificity for HCC diagnosis from 62 % for DCP only to 92 % for the DCP/NX-DCP ratio, although with a minimal reduction in sensitivity (18). Similarly, PIVKA-II is also a good serum biomarker for monitoring the treatment response and prediction of prognosis. As reported in previous studies, elevated serum levels of des-γ-carboxyprothrombin levels are associated with a greater activity of tumour proliferation and increased risks of metastasis in the intrahepatic region, capsular infiltration and vascular invasion, which contribute to poor prognosis in HCC (13). Diseases causing the deficiency of vitamin K, ingestion of warfarin and other antibiotics, malnutrition and alcoholic liver diseases limit the diagnostic value and clinical utility of DCP as a marker of HCC as they cause its elevation also in patients without HCC (13).

AFP-L3

AFP-L3, also referred to as *lens culinaris* agglutinin-reactive AFP, is an AFP glycoform fucosylated for the detection of early-stage HCC. Conventional AFPL3 assay recommends an AFP level > 10 ng/mL for detection, whilst the use of a highly sensitive assay for AFP-L3 (hs-AFP-L3) increases the potential and enables measurements to be conducted in patients with AFP levels as low as 2 ng/mL (19). Japan remains the only country where AFP-L3 has been included and can be measured in a routine clinical setting. Interestingly though, this isoform is generally considered to be more specific but less sensitive in the diagnosis of HCC compared to AFP (20) This assay provides and is used for an early diagnosis of HCC in cases with tumour diameter < 2 cm while its performance worsens with clinical stages (21). Elevated levels of AFP-L3 can be measured in the sera of nearly one-third of patients with small HCC (< 3 cm) when cut-off levels are provided in range of 10–15 %. With a cut-off value above 15 %, the proportion of AFP-L3 to total AFP demonstrates a sensitivity of 75–97 % and specificity of 90–92 % for the diagnosis of HCC (22). AFP-L3 may be elevated in some patients with an appearance of a mass in the liver and therefore cannot be recommended for general use. Moreover, this test requires special equipment and is expensive (21).

Glypican-3 (GCP3)

Glypican-3 is a membrane proteoglycan anchored on a heparin sulphate. There is a significant up-regulation of GCP-3 in HCC compared to other complications involving the liver. GCP-3 is considered an independent biomarker that is superior to alpha-fetoprotein (AFP) in the diagnosis of HCC. The amino-terminal serves as a signal in the detection of HCC even in special cases where alpha-fetoprotein (AFP) and des-γ-carboxyprothrombin (DCP) have negative levels. GCP-3 has a specificity > 95 %, while its sensitivity is lower, namely 55.2 % (23). GPC-3 is correlated with the development of HCC and its progression; with a significant elevation in patients with HCC compared to those with pre-neoplastic lesions. Significantly, the levels of GPC-3 are more commonly increased compared to those of alpha fetoprotein (AFP) in patients with HCC, which is very apparent in patients with small-sized HCC (24).

MicroRNA

MicroRNAs (miRs) are non-coding RNAs in smaller units that can post-transcriptionally regulate the expression of genes (25). Expression of these miRs has been found to be associated with tumorigenesis. The downregulation of miRNA-122, the most abundant form of miRNA in the liver, acts as a tumour suppressor gene for hepatocarcinogenesis (26). Changes in the expression of miRs like miR-122 have been associated with poor prognosis of HCC due to the lack of suppression by means of angiogenesis (27). The stability and resistance to endogenous RNase activity are of significant advantage when using miRNAs as diagnostic tools as these factors allow research samples to be frozen and stored

with little or no degradation (28). Although efforts are underway to procure uniformity in characterization of miRNA molecules, the challenges with miRNA analyses arise from many distinct annotations, (19).

Golgi-protein 73

Golgi-protein 73 is a trans-membrane protein localized in the Golgi complex. The Golgi protein is expressed primarily in the cells of the epithelial lineage, albeit it is expressed in smaller quantities in the hepatocytes of a healthy liver. GP73 expression is unregulated in the livers of individuals infected with HBV or HCV viruses, especially in cases with cirrhosis, or focal nodular hyperplasia, and it gets significantly elevated in HCC (18). Although high levels of serum GP73 are exhibited by several classes of tumour cell types that can be used in the diagnosis of HCC (29), GP73 has been shown to be a useful biomarker for distinguishing between benign and malignant tumours as well as between liver and non-liver malignancies due to its substantially elevated serum levels in HCC patients. GP73 has a great potential to be used as a serum marker in diagnosis, surveillance, recurrence and post-operative management of HCC (30). GP73 remains undetectable in small tumour lesions. A significant challenge, however, is the over-reliance on western blot analysis for accurately measuring isoforms related to HCC (19).

Conclusion

Current guidelines emphasize the clinical significance of HCC biomarkers. Over the past decades, straightforward approaches have been adopted in developing novel biomarkers in routine surveillance and non-invasive diagnosis of HCC. Unfortunately, no single biomarker has demonstrated the requisite level of sensitivity and specificity of HCC. Given the heterogeneous nature of both human subjects and tumours, the prospect of identifying a sole ideal biomarker for HCC remains uncertain. Although AFP remains the most widely used biomarker in most clinical settings, relying solely on it for diagnostic accuracy is not adequate. However, the combined diagnostic sensitivity of AFP with AFP-L3 or DCP has shown superior performance as compared to assessing individual biomarkers alone (9, 20) The latter approach is currently being used in Asia, especially in Japan. Several studies conducted over the past years demonstrated the clinical utility of novel biomarkers such as miRNA, GP73, and GCP3 in the early diagnosis of HCC. Best strategy for advancing the diagnosis of HCC is to have a detailed characterization of these novel biomarkers. However, these biomarkers have shown to have a capacity to improve the diagnostic precision. Further studies need to prioritize the predictive ability and diagnostic utility of combined biomarkers in order to maximize the benefits for patients with HCC. Additionally, there is the need to refine the understanding of their mechanism of action and potential role in clinical practice. The anticipation is that novel biomarkers can enhance the clinical practice and help improve the patient care.

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