Int J Clin Exp Pathol 2024;17(11):383-395
www.ijcep.com /ISSN:1936-2625/IJCEP0157546

Review Article

Alpha-fetoprotein, glypican-3, and kininogen-1 as biomarkers for the diagnosis of hepatocellular carcinoma

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Received May 3, 2024; Accepted September 23, 2024; Epub November 15, 2024; Published November 30, 2024

Abstract: The hepatocarcinoma (HCC) is the most important liver tumor. It represents 90% of liver cancer cases. One of the main problems is the limited prompt cancer diagnosis and the advanced stages where the chances of treatment are limited. The main diagnostic methods for HCC are imaging techniques and liver biopsy. With advances in technology, proteins as significant diagnostic biomarkers have increased. The objective of this review is to describe the role of Alpha-fetoprotein (AFP), Glipican 3 (GPC-3), and Kininogen 1 (KNG-1) as biomarkers for the diagnosis of hepatocellular carcinoma. A systematic search of studies was carried out in the literature and the diagnostic values of these proteins were compared. The results showed that the combined use of biomarkers increases the diagnostic capacity for the detection of hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, liver cancer, biomarkers, alpha-fetoprotein, glypican 3, kininogen 1

Introduction

Hepatocellular carcinoma (HCC) is a malignant tumor in hepatocytes. It is the most important primary liver tumor. It comprises approximately 80% of liver cancer cases worldwide [1]. There are various types of primary liver cancer: intrahepatic cholangiocarcinoma (ICC), angiosarcoma, hepatoblastoma, hemangioma, and hepatic adenoma. HCC and ICC are the most prevalent [2].

Liver cancer is in the seventh place of incidence worldwide, observing a large increase annually [3]. It ranks fourth as the cause of death from cancer in the world. It is estimated that there are more than half a million cases per year. This makes it the second most lethal malignant tumor [3]. This tumor has a high incidence in Asian countries and sub-Saharan Africa because of hepatitis viruses [4]. The diagnosis in western countries is increasing. A decrease was observed in eastern countries. In recent decades, an increase in the incidence of HCC has been observed in the United States because of different risk factors such as infection by hepatitis C virus (HCV) and alcoholic and non-alcoholic liver diseases [5]. The most affected gender is men, with a ratio of 2:1 compared to women. Regarding the average age of diagnosis, in most cases they were between 30 and 60 years [6]. This differs in different populations, even within the same continent, such is the case of Africa where there is a significant difference in the average age of diagnosis between Egypt (58 years) and African countries (46 years). In the United States the average age of diagnosis is 60 to 64 years in men and 65 to 69 years in women [7].

Risk factors for HCC

HCC has a multifactorial etiology, the main ones are hepatitis B and C viruses (HBV, HCV),

https://doi.org/10.62347/QSII4050

followed by alcoholic liver disease and metabolic dysfunction-associated steatotic liver disease (MASLD) [8]. The largest proportion of cases is associated with liver cirrhosis, and less frequently it occurs because the consumption of foods contaminated with aflatoxins, congenital diseases such as hereditary tyrosinemia, Wilson's disease, alpha-1 antitrypsin deficiency, hereditary hemochromatosis, and autoimmune hepatitis [9].

In countries such as the United States, an increase in metabolic syndrome, diabetes mellitus, overweight, and obesity that are risk factors involved in hepatocarcinogenesis. In countries with a high incidence of HBV and HCV, it has been observed that these viruses are responsible for 75-80% of HCC cases. The increase in HCC was because the HCV is high in developing countries. Approximately 60% was observed in developing countries. In developed countries, it is barely 23% [10].

The majority of HCC cases worldwide are caused by HBV, with an overall attributable risk of approximately 40%, followed by hepatitis C virus (HCV). This accounts for 28-30% of cases, with significant geographic variations between the eastern and western world [11]. The role of the HBV and HCV viruses in hepatocarcinogenesis is associated with chronic inflammation, fibrosis, and cirrhosis. This involves the cellular homeostasis alteration caused by the integration of viral DNA into the host genome [12].

Another risk factor for the development of HCC is MASLD. This disease is characterized by the accumulation of lipids in the liver, causing inflammation and damage to hepatocytes. Excess hepatic lipid stores are called steatosis. It can lead to the progression of metabolic steatohepatitis (MeSH) formerly called non-alcoholic steatohepatitis (NASH), followed by liver fibrosis and leads to HCC [13].

HCC diagnosis

The life prognosis in HCC depends on the stage tumor. Timely diagnosis is an important goal of the survival in affected patients. The treatment depends to a great extent on the stage of the tumor [14]. The different clinical practice guidelines for HCC used around the world, describe different diagnostic methods recommended for the detection of this neoplasm according to the stage and size of the tumor [15]. The Asian Pacific Association for the Study of the Liver (APASL), the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Diseases (AASLD) [16], and the Clinical Practice Guide for the Prevention, Diagnosis, and Treatment of hepatocarcinoma in Mexico, describe liver biopsy, imaging techniques, and alpha-fetoprotein as diagnostic methods for HCC.

The diagnosis methods of HCC include invasive and non-invasive. In the context of liver cirrhosis, imaging techniques considered within non-invasive methods are used. The different societies maintain that invasive criteria, the use of liver biopsy, is used when there is no conclusive diagnosis of HCC in imaging techniques [17]. Liver biopsy has been considered as the gold standard in the diagnosis of HCC [18]. A liver biopsy has some limitations. The tumors are heterogeneous and there is no histological pattern. It can generate sampling errors, giving rise to a false negative rate of around 30% [19]. Imaging techniques are based on the vascular findings of HCC, understanding the venous processes, pathophysiology, and carcinogenesis are important for correct imaging diagnosis and treatment [20].

The radiological tests used for the diagnosis of HCC are computed tomography (CT), magnetic resonance imaging (MRI), and abdominal ultrasound (USG). Multistage CT is a second-line imaging modality that allows visualization of HCC solid tumors without histologic confirmation [21]. This non-invasive technique measures the X-ray attenuation properties of different tissues. It provides high-resolution three-dimensional images in a short time, making it a valuable diagnostic tool [22]. Trials have shown this method to be less cost-effective compared to other imaging techniques A randomized clinical trial (RCT) of 163 patients demonstrated that multiphasic CT has a similar sensitivity to ultrasound (66.4% vs. 71.4%, respectively) [23]. Sensitivity and specificity for early diagnosis of HCC remain low, showing additional risk of exposure and contrast-induced nephrotoxicity [24]. Magnetic resonance imaging (MRI) has become a non-invasive method for the diagnosis of neoplasms. It has a submillimeter spatial resolution, high anatomical contrast, and excellent soft tissue differentiation. In some cases

Table 1. Biomarker development and validation phases in cancer research

Phase	Study design	Objectives
I	Preclinical.	Identify clinical biomarkers.
II	Clinical-exploratory.	Disease detection.
III	Observational-retrospective.	Detection of cancer in asymptomatic stages.
IV	Observational-prospective.	Extent and characteristics of the disease. False referral rate.
V	Trial-control.	Impact on survival. Tumor progression.

Note: Adapted on "Biomarkers in Hepatocellular Carcinoma: Diagnosis, Prognosis and Treatment Response Assessment" [34].

contrast agents required (CA) based on gadolinium to improve differences in signals between diseased areas and normal tissue [25]. Image resolution depends on echo amplitude and proton density, and to a lesser extent on factors such as flow, perfusion, diffusion, and magnetization transfer [23]. There are disadvantages to the use of MRI, one of which is the high diagnostic sensitivity in large tumors, difficulty in diagnosing small lobes of pseudolesions or hypervascular nodules of non-malignant origin, and high cost compared to other methods [24]. Abdominal ultrasound is a medical diagnostic technique that is based on the action of ultrasound waves generating images through ultrasound beams (echoes), reflected by body structures [26]. It is an economical, real-time, and non-invasive method, and the most used for the detection of liver diseases [27]. Abdominal ultrasound has a sensitivity of 84% in the detection of any stage of HCC. In early stages, it reaches a sensitivity of around 47% (31). A contrast ultrasound reaches a sensitivity of 95% and a specificity of 98.1% [28].

HCC biomarkers

The Biomarker Definitions Working Group of the National Institutes of Health defines biomarkers as "molecules or substances that measure, evaluate, and act as indicators of normal biological processes, pathogenic processes, or pharmacological responses to interventions" [29]. The most used biological samples are whole blood, erythrocytes, plasma, serum, urine, nails, saliva, feces, and samples of different tissues [30]. Biomarkers can be prognostic or predictive. Prognostic biomarkers are those that provide information about the presence or progression of a disease. Predictive biomarkers report on the probable response to treatment [31]. Through the development of multiple biotechnologies (omic data, genomics, epigenomics, transcriptomics, proteomics, metabolomics, and metagenomics), HCC diagnostic biomarkers have been discovered. This has been validated in different clinical trials [32]. The Cancer Institute Early Research Network (EDRN) classifies biomarkers into 5 phases of development and validation. Most studies have reached phase 2. Phase 3 studies have contributed to the elimination of bias inclusion and verification [33]. **Table 1**, describes the development and validation phases of the biomarkers.

Alpha-fetoprotein (AFP) is the only HCC biomarker that has been validated for clinical use. Biomarkers have only been evaluated in a few studies for early detection of HCC [34]. According to the EDRN, 13 proteins have been found as biomarkers for liver cancer, described in **Table 2**. Of these 13 proteins, we have focused in this review on the description of only 3 (AFP, GPC3, and KNG1). We have found good diagnostic performance in their combination. These are preliminary data that we have to publish.

Validated biomarker: AFP

AFP is a 72 kDa oncofetal glycoprotein, made up of 591 amino acids [35]. It is responsible for transporting steroids, bilirubin, fatty acids, flavonoids, heavy metals, dioxin, and drugs [36]. It is produced during fetal life, initially in the yolk sac, and then in the fetal liver. In adults its synthesis is repressed [37]. The gene that codes for this protein is found in 4q11-13 with 15 exons and 14 introns. It is a member of the albumin family and is highly homologous to serum albumin, alpha albumin, and vitamin D-binding proteins [38]. AFP plays an important role in hepatocarcinogenesis, regulates

Table 2. Candidate biomarkers for the diagnosis of HCC

Biomarker	Description	Development phase	Sensitivity	Specificity	Area under the ROC curve	References
Alpha-fetoprotein (AFP)	Oncofetal glycoprotein is responsible for transporting steroids, bilirubin, and fatty acids. It is expressed in conditions such as pregnancy and neoplasias such as HCC.	V	39-64%	76-97%	0.75-0.82	[80]
Lens culinary agglutinin-reactive fraction of fetoprotein (AFP-L3)	AFP subtype, culinary lens agglutinin-reactive fetoprotein fraction (AFP-L3), is derived from cancerous hepatocytes and is considered specific for HCC.	III	34%	92%	0.75	[81]
Golgi protein 73 (GP73)	Type II Golgi-localized integral membrane protein is expressed in epithelial cells of human tissues, and its expression is increased in samples from HCC patients.	I	69%	75%	0.79	[82]
Des-carboxyprothrombin (DCP)	Abnormal prothrombin is produced by hepatocytes because of vitamin K deficiency. Levels are elevated in the serum of HCC patients.	II	48-62%	81-91%	0.76	[83]
Dickkopf-1 (DKK-1)	Wnt signaling regulator, associated with carcinogenesis, metastasis, recurrence, and poor prognosis in HCC.	II	80.5%	53.2%	0.70	[84]
Osteopontin (OPN)	The multifunctional phosphorylated glycoprotein, expressed in T lymphocytes, macrophages, dendritic cells, and osteoclasts.	II	81.3%	87.4%	0.91	[85]
Midkine (MDK)	Low molecular weight heparin-binding basic growth factor. It has an important role in carcinogenesis-related activities, such as proliferation, migration, antiapoptosis, mitogenesis, transformation, and angiogenesis, in many types of solid tumors, including HCC.	II	86%	75.4%	0.92	[86]
Glypican 3 (GPC-3)	Heparin sulfate transmembrane proteoglycan, an inhibitor of apoptosis, is closely linked to the growth, proliferation, invasion, and metastasis of cancer cells.	II	75%	87%	0.79	[87]
α-L-fucosidase (AFU)	Lysosomal enzyme is detected in most mammalian cells and is associated with the degradation of fucoglycoconjugates containing fucose. The expression has been found in HCC patients.	II	85.2%	98.9%	0.96	[65]
Squamous cell carcinoma antigen (SCCA)	Protects tumor cells from apoptosis. SCCA expression, and AFP production are observed in the presence of HCC.	II	84%	49%	0.70	[88]
Kininogen 1 (KNG-1)	It plays an important role in many pathophysiological processes which include fibrinolysis, thrombosis, inflammation, and oncogenesis.	I	Data for early-stage HCC not available	Data for early-stage HCC not available	Data for early-stage HCC not available	[55]
Annexin A2	Calcium-dependent phospholipid-binding protein is present in endothelial cells and on the surface of most epithelial cells, it is expressed during hepatocarcinogenesis.	II	81.7%	68.3%	0.87	[89]
Soluble urokinase plasminogen activator receptor (suPAR)	It is expressed in endothelial and epithelial cells where it is activated in the tissue repair process. It is elevated in patients with liver failure, colon cancer, and HCC.	III	76%	90.4%	0.95	[90]

Note: The table describes 13 proteins that are useful as biomarkers of liver cancer. Adapted from the Early Detection Research Network (EDRN) of the National Cancer Institute of the United States of America.

the expression of oncogenes, inhibits apoptosis, promotes the growth of cancer cells, and improves drug resistance [39]. In some studies, AFP acts as an intracellular signaling molecule that binds to key proteins involved in cell growth and apoptosis pathways. This protein can block RA-RAR signaling and the caspase-3 cascade [40]. The studies demonstrated that AFP promotes metastasis. The overexpression of AFP in tumor cells has a fundamental role in the molecular mechanisms of the PI3K/AKT signaling pathway, and is associated with cell metastasis [41]. In the diagnosis of HCC, international guidelines such as JSH and APASL, recommend surveillance of HCC based on serum AFP determination, accompanied by ultrasonography. The current American and European guidelines, the AASLD, and the EASL-EORTC guidelines, do not recommend AFP as a screening program because of low sensitivity and cost-benefit issues [42]. Another present problem for the diagnosis of HCC from AFP is because of the cut-off point used for detection. In a meta-analysis the diagnostic specificity values increased considerably as the cut-off point increased. When increasing to a cut-off value of 200 ng/ml, a specificity of 98% was observed. At the cut-off point of 400 ng/ml the specificity increased to 99%. An AUROC of 0.93 was observed for both cut-offs respectively [43]. The AFP is the only tumor biomarker used for the diagnosis of HCC. The lack of diagnostic accuracy has made it an additional method for the detection of HCC. It has been shown that AFP is strongly related to the aggressiveness of the tumor [44]. AFP is a diagnostic biomarker. The characteristics as a modulator of cell growth in the neoplastic process has made it a target biomarker for the treatment of HCC [45].

Glypican 3 (GPC3)

GPC3 is a 60-70 kDa heparin sulfate transmembrane proteoglycan attached to the cell membrane surface by a glycosylphosphatidylinositol (GPI) anchor, and carboxyl-terminal modified with a heparan sulfate side chain [46]. It is encoded by the GPC3 gene on the X chromosome (Xq26.2). GPC3 has a cleavage site between Arg358 and Ser359 for the furin protease, resulting in a 40 kDa N-terminal subunit and a 30 kDa C-terminal subunit [47]. GPC3 belongs to the family of integral membrane proteoglycans, which includes six members (GPC1-GPC6) [48]. All GPC proteins are highly expressed during embryonic development: GPC1 is expressed in bone, bone marrow, muscle, epithelium, and kidney. GPC2 is expressed in the nervous system. GPC3 and GPC6 are expressed on embryonic cell surfaces. GPC4 is expressed in the brain, kidney, and lung. GPC5 is expressed in the brain, lung, liver, kidney, and extremities [49].

Studies have shown that GPC3 plays an important role in regulating cell cycle division and growth through the Wnt (Wingless-related integration site), hedgehog (Hh), bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) signaling pathways [50]. GPC3 induces apoptosis by anchoring to cell membrane proteins, acting as an inhibitor of cell proliferation [51]. GPC3 functions as a co-receptor for ligands of the Wnt and FGF pathways through heparan sulfate side chains, facilitating the activation of signaling pathways involved in HCC development. GPC3 is involved in the canonical Wnt/β-catenin signaling pathway, promoting cell growth [52]. Studies have revealed the importance of GPC3 in regulating the tumor microenvironment and cancer metastasis through epithelial-mesenchymal transition (EMT), a key process in cell invasion [47].

GPC3 can be released from the cell surface through the GPI anchor, allowing the protein to be found in serum [52]. GPC3 expression is absent in tissues from healthy patients or pathological samples of fatty liver, cirrhosis, hepatitis, or lesions [8]. Studies have linked GPC3 as a biomarker for lung carcinoma, severe pneumonia, and acute respiratory distress syndrome (ARDS) [48]. One study analyzed 240 HCC samples stained with GPC3 and found that 48.3% of cases showed positive staining with a diagnostic accuracy of 87.5% [51]. GPC3 is associated with therapeutic methods for HCC treatment because its properties as an optimal target for drug delivery. It is specific to HCC cancer cells, bound to the cell membrane, and protrudes from the cell compartment, facilitating the binding of specific anti-GPC3 antibodies to tumor cells [53]. Recent studies support the development of antibody-based therapeutic strategies, including immunotoxins and ADCs targeting GPC3 for HCC treatment. For example, a study in 2020, demonstrated that CAR T cells (hYP7) could induce sustained

Table 3. Diagnostic values of KNG-1 in different pathologies

	Sample	*Pathology*	*Sensibility*	*Specificity*	*AUROC*	*Reference*
KNG-1	Serum	HCC	12.8%	Not available	0.64	[79]
KNG-1	Plasma	Lung squamous cell carcinoma (LUSC)	74%	75%	0.81	[15]
KNG-1	Urine	(LUSC)	90%	59%	0.81	[15]
KNG-1	Bronchoalveolar lavage fluid (BALF)	(LUSC)	92%	73%	0.91	[15]
KNG-1	Serum	Polycystic ovary syndrome	79%	95%	0.90	[91]

Note: A comparison is made in the table of the diagnostic values of KNG-1 in different pathologies. Abbreviations: KNG-1, Kininogen 1; HCC, Cellular hepatocarcinoma; AUROC, Area under the curve.

tumor regression in HCC-bearing mice, leading to the development of CAR T cell therapy targeting GPC3 for HCC patients [54].

Kininogen 1 (KNG-1)

Kininogen belongs to the superfamily of cystatins, multifunctional proteins with multiple domains that maintain normal physiological conditions in humans. It plays an important role in many pathophysiological processes, including fibrinolysis, thrombosis, inflammation, and oncogenesis [55]. Kininogen-1 is encoded by the KNG1 gene and belongs to the plasma kallikrein-kinin system, comprising factor XII, prekallikrein, and factor XI. In this system, kininogen acts as a bradykinin-releasing factor, a potent inflammatory mediator. The KNG1 gene generates high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK), both modular plasma proteins. This gene is located on chromosome 3q27. Human KNG HMW and LMW are made up of 644 and 427 amino acids, respectively [56]. KNG-1 stands out for its antiangiogenic properties and its inhibitory action on endothelial cell proliferation. This suggested that decreased expression in serum and plasma contributes to the survival of cancer cells [57].

Proteomic serum profiles were performed using mass spectrometry. Multidimensional analysis of spectra showed algorithms capable of distinguishing the expression profiles of specific proteins in serum samples. There were 100 peaks detected per single spectrum, with three peaks belonging to kininogen-1 and thymosin-β4. Kininogen-1 mRNA was significantly downregulated in cirrhotic livers [58]. Studies have identified KNG-1 as a significant marker in the early stages of colorectal cancer using proteomic or immunohistochemical techniques [57]. It has been associated as a biomarker for ovarian cancer [58], oral cancer [59], and diabetic nephropathy [60]. There is little evidence of its use as a marker for HCC. In 2020, a study discovered prognostic biomarkers for HCC in patients diagnosed with liver cirrhosis. These results show that, KNG-1 was observed to be present in HCC and a significant candidate for therapeutic targeting in different types of cancer [61]. KNG1 has been identified as a favorable prognostic marker in liver cancer. A study using Kaplan-Meier survival curves and the log-rank test showed that high expression of KNG1 was a good prognostic predictor for patients with HCC [62]. Another study mentioned that low expression of KNG1 in patients with HCC increases the vitality of cancer cells and plays a crucial role in carcinogenesis. This indicated a direct relationship between low KNG1 levels and the severity of liver function impairment [63]. Despite the evidence presented on the use of KNG-1 as an HCC biomarker, there are few studies on the diagnostic capacity of this protein. **Table 3** summarizes a comparison of the diagnostic values of KNG-1 in different pathologies.

Combined HCC biomarkers (in tandem)

In the last two decades, basic and clinical studies on AFP have made some progress. In 2020, a study conducted a meta-analysis where they showed that the combination of AFP-Lens Culinars Alpha-Fetoprotein (AFP-L3) and DCP had good diagnostic performance in the detection of HCC. The sensitivity and specificity of this combination were 88% and 79%, respectively with an AUC of 0.91 [64]. Studies in 2021, showed that the combination of AFP and α-L-fucosidase (AFU) improved the diagnostic specificity for early-stage HCC with AUC of 0.77, sensitivity of 52.5%, and specificity of 91.6% [65]. In accordance with the previous studies [66], it can be seen that the diagnostic significance of AFP can be improved by combining it with diagnostic markers (**Table 4**). An extensive investi-

Table 4. Sensitivity and specificity of AFP combined with other biomarkers

HCC Biomarkers in tandem	*Sensitivity*	*Specificity*	*AUROC*	*Reference*
AFP + GP73	89.2%	85.2%	0.96	[75]
AFP + OPN	75.0%	72.0%	0.73	[92]
AFP + DKK-1	78.4%	72.5%	0.75	[92]
AFP + PIVKA-II	87.5%	92.5%	0.94	[76]
AFP + AFU	52.5%	91.6%	0.77	[65]
AFP + PIVKA-II + OPN	75.5%	70.5%	0.73	[92]
AFP + PIVKA-II + DKK-1	79.3%	69.9%	0.74	[92]
AFP + OPN + DKK-1	84.6%	56.5%	0.75	[92]
AFP, AFP-L3 y DCP	81%-93%	69%-87%	0.88	[66]
AFP + PIVKA-II + OPN + DKK-1	85.1%	54.9%	0.70	[92]

Note: The table describes the sensitivity and specificity of AFP combined with other biomarkers. The diagnostic significant of AFP can be improved by combining it with other diagnostic markers. Abbreviations: AFP, Alpha-fetoprotein; GP73, Golgi-73 protein; DKK-1, Dickkopf-1; PIVKA-II, Prothrombin induced by vitamin K deficiency or II antagonist; AFU, α-L-fucosidase; OPN, Osteopontin; AFP-L3, Lens culinars Alpha-fetoprotein; DCP, Des-carboxyprothrombin.

Table 5. Sensitivity and specificity of GPC3 combined with other biomarkers

Biomarkers	*Sensitivity*	*Specificity*	*AUROC*	*Reference*
GPC3 + AFP	91%	70%	0.92	[93]
GPC3 + PIVKA II	84.2%	57.5%	0.83	[94]
GPC3 + AFP + GP73	91%	84%	0.92	[67]
GPC3 + VEGF	60%	100%	0.80	[69]
GPC3 + GP73	78%	60%	0.64	[69]
GPC3 + VEGF + GP73	83.3%	60%	0.74	[69]

Note: The table describes the sensitivity and specificity of the combined use of GPC3 (Glypican 3) with the following biomarkers: PIVKA II, Prothrombin induced by vitamin K deficiency or antagonist II; AFP, alpha-fetoprotein; GP73, Golgi-73 protein; VEGF, Vascular endothelial growth factor.

gation in multicenter studies with larger cohorts and long-term evaluation is required to confirm clinical utility. The development of algorithms with serum markers and non-invasive imaging techniques to improve early and accurate diagnosis of HCC.

Studies conducted in 2020 evaluated the diagnostic value of the combination of GP73, GPC3, and AFP. This combination proved to be accurate with a 65.9% probability of distinguishing benign and malignant liver lesions. The results showed a sensitivity of 91%, specificity of 84%, and an AUROC of 0.92. This considerably improved diagnostic performance [67]. Studies analyzed the combination of three biomarkers: AFP, PIVKA-II, and GPC-3. This study included 349 patients (200 with cirrhosis and 149 with HCC). The results showed an AUC of 0.79 for PIVKA-II, 0.73 for AFP, and 0.63 for GPC-3. The combination of AFP and PIVKA-II presented an AUC of 0.82. The addition of GPC-3 did not improve diagnostic performance [68]. A 2023 study evaluated the clinical utility of GPC3, vascular endothelial growth factor (VEGF), and GP73 in serum samples. This study included 50 patients with HCC, 50 with liver cirrhosis, and 20 healthy subjects with no history of liver disease. The results showed that the combination of VEGF and GP73 had a sensitivity of 85% and specificity of 80%, with an AUC of 0.59. The combination of GPC3 and VEGF had a sensitivity of 60% and specificity of 100%. The combination of GPC3 and GP73 had a sensitivity of 78% and specificity of 60%. A triple combination showed a sensitivity of 83.3% and a specificity of 60%, with an AUC of 0.742. The combination of VEGF and GP73 has a promising role in the diagnosis of HCC in cirrhotic patients by a non-invasive method [69].

In 2023, a prospective study was conducted involving 154 patients previously diagnosed with liver cirrhosis. These patients were divided into two groups. The first group included 95 patients with a diagnosis of HCC based on clinical evaluation and imaging. The second group included 59 patients without HCC. The main objective was to compare the efficacy of GPC3 and CK19 as independent biomarkers for predicting HCC using a statistical model called the "GALKA" score. The results showed that AFP performed better as a biomarker for predicting HCC compared to GPC3, DPC, and CK19. No significant differences were found between AFP and AFP-L3. Both AFP and AFP-L3 performed better than GPC3 ($P < 0.0001$) and CK-19 ($P < 0.0001$). GPC3 had a better performance in predicting HCC than CK-19 ($P = 0.0067$). **Table 5** summarizes the combined use of GPC3 with biomarkers.

Table 6. Sensitivity and specificity of KNG1 combined with other biomarkers

Biomarkers	*Sensitivity*	*Specificity*	*AUROC*	*Reference*
KNG1 + APOC3 + PON1	100%	85%	0.95	[70]
KNG1 + CEA	21.6%	92.9%	Not available	[71]
KNG1 + ANXA2 + HSPA5 + PRDX2	51.6%	88.6%	Not available	[72]
KNG1 + ANXA2 + FLNA + HSPA5 + PRDX2 + TIMP1 + YWHAB	75.8%	96.2%	Not available	[72]
KNG1 + MMP1 + ANXA2 + HSPA5	96.7%	79.7%	Not available	[72]

Note: The table describes the sensitivity and specificity of the combined use of KNG 1 (Kininogen 1) with the following biomarkers: APCO3, apolipoprotein C-III; PON1, paraoxonase 1; CEA, carcinoembryonic antigen; ANXA2, annexin A2; HSPA5, heat shock 70 kDa protein 5; PRDX2, peroxiredoxin 2; FLNA, actin-binding protein; TIMP1, tissue inhibitor of matrix metalloprotease 1; YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; MMP1, matrix metalloproteinase 1.

Regarding KNG 1, few studies show the combination of this biomarker with other existing. In 2016, the sensitivity and specificity of KNG 1 accompanied by proteins such as apolipoprotein C-III (APOC3) and paraoxonase 1 (PON1) for the diagnosis of Yin-heat deficiency syndrome (YDH) was evaluated. Results showed a sensitivity of 100% and a specificity of 85%, with an AUC of 0.950 [70].

In studies for the early detection of adenocarcinoma and colorectal cancer, proteomic profiles of serum samples from 35 healthy patients, 35 patients with advanced colorectal adenoma, and 40 patients with colorectal cancer were compared. The results showed that KNG-1 had a sensitivity of 63.6% and a specificity of 65.8%. When used in tandem with carcinoembryonic antigen (CEA), the sensitivity was only 21.68%, but the specificity increased significantly to 92.9% [71].

A study evaluated diagnostic biomarkers in saliva for the early detection of oral squamous cell carcinoma (OSCC). A total of 302 protein biomarkers were identified in the data. Only 28 could be quantified. Using a logistic regression analysis, combinations were established for a panel of these biomarkers, including: annexin A2 (ANXA2), heat shock 70 kDa protein 5 (HSPA5), peroxiredoxin 2 (PRDX2) and Kininogen 1 (KNG1) with a sensitivity of 51.6% and specificity of 88.7%. In 2016, the combination of four proteins: matrix metalloproteinase 1 (MMP1), KNG1, ANXA2, and HSPA5, presented a better diagnostic performance for oral squamous cell carcinoma, with a sensitivity of 96.7% and a specificity of 79.7% [72], **Table 6**.

Discussion

There are different diagnostic methods for hepatocellular carcinoma. There is not a sufficiently sensitive method to detect this type of cancer in its early stages. The study of biomarkers has emerged, aiming to find an accurate and safe diagnostic method for the patient. The main objective of this review was to describe current studies on AFP, GPC-3, and KNG-1 as diagnostic biomarkers for HCC, alone or in combination. This review describes the cellular processes in which these proteins are involved and their expression in different neoplastic tissues. We show the sensitivity and specificity values in comparison with other biomarkers for HCC. Within the data reviewed, we found that these three proteins are present in patients with HCC and differ in their diagnostic capacity for this neoplasia. In clinical practice, the most used biomarker is AFP. Its low sensitivity and specificity may present with values within normal ranges (negative) in patients with HCC [73]. Studies reveal that increasing the AFP cut-off point from 20-100 ng/mL to > 200 ng/mL and > 400 ng/mL considerably increases the specificity and capacity of the diagnostic test (AUROC 0.83 vs. 0.93) [43]. Researchers have described that including a lower cut-off point (10-20 ng/mL) results in a sensitivity of only 60% [74]. The combination of two or more biomarkers considerably increases the sensitivity and specificity parameters. The combinations with the best diagnostic capacity are AFP and GP73 (Sensitivity 89.2%, specificity 85.2%, and AUROC 0.96) [75], AFP and PIVKA-II (Sensitivity 87.5%, specificity 92.5%, and AUROC 0.94) [76], and AFP, AFP-L3, and DCP (Sensitivity 88.0%, specificity 79%, AUROC 0.90) [64] compared to AFP alone (Sensitivity 64.2%, specificity 90.2%, and AUROC 0.77). Elevated AFP levels are associated with invasive histological morphology. AFP negativity is a predictive factor for eligibility in liver transplantation [77]. Despite AFP's important role in HCC, there are associations to be resolved.

Regarding GPC-3, research has described serum and tissue overexpression in patients with HCC and a null expression in healthy people, making it a reliable biomarker for this pathology [52]. The expression of GPC-3 seems to be independent of the size of the tumor. Some studies have revealed sensitivity percentages of 55.2% and specificity of 84.2%. The combined use of AFP and GPC3 manages to increase the sensitivity to 76% in early-stage tumors [8]. Data collected showed that increasing the combination of proteins (GPC3, AFP, and GP73) increases the AUROC to 0.92, with a sensitivity and specificity of 91% and 84%, respectively [67]. Studies demonstrated a less accurate diagnosis compared to AFP, attributing it to the fact that GPC3 is not related to tumor size and is better expressed in early stages [78].

Regarding KNG-1, the results showed that it works as a biomarker for different pathologies, with significant diagnostic capacity for lung squamous cell carcinoma (sensitivity 74%, specificity 75%, and AUROC 0.81) [15]. He presence of this protein in serum, plasma, and urine was observed. Regarding the diagnosis of HCC, there are few studies that relate it as a biomarker, for example a study reported a sensitivity of 12.8% and an AUROC of 0.64 [79]. Research has linked it as a survival marker for HCC patients, where high levels of KNG-1 are associated with a better prognosis [63].

Statistic analysis

No statistical analysis was performed. All data presented are derived from the scientific articles reviewed.

Acknowledgements

The authors would like to thank the Instituto de Salud Pública of the Universidad Veracruzana for its support.

Disclosure of conflict of interest

None.

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