Biomarkers in Diagnosis and Prediction of Hepatocellular Carcinoma Recurrence (Review)

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S.I. Malov, MD, PhD, Assistant, Department of Infectious Diseases1; Senior Researcher, Central Scientific Research Laboratory2; I.V. Malov, MD, DSc, Professor, Head of the Department of Infectious Diseases1; V.V. Dvornichenko, MD, DSc, Professor, Head of the Department of Oncology and Radiotherapy1; Head of the Oncology Department2; P.N. Marche, PhD, Professor, Vice Director of Research Center3; T. Decaens, PhD, Professor, Research Director, Laboratory Head of Department of Hepatology and Gastroenterology4; Z. Macek-Jilkova, PhD, Researcher of Department of Hepatology and Gastroenterology4; N.D. Yushchuk, MD, DSc, Professor, Academician of the Russian Academy of Sciences, Head of the Department of Infectious Diseases and Epidemiology5

1Irkutsk State Medical University, 1 Krasnogo Vosstaniya St., Irkutsk, 664003, Russia; 2Irkutsk State Medical Academy of Postgraduate Education, a Branch of the Russian Medical Academy of Continuing Professional Education, 100 Yubileyny Microdistrict, Irkutsk, 664049, Russia; 3Institute for Advanced Biosciences, Research Center Inserm U1209, CNRS 5309, Univ. Grenoble-Alpes, La Tronche, 38700, France; 4CHU-Grenoble, Clinique Universitaire d’Hépato-Gastroentérologie, La Tronche, 38700, France; 5A.I. Yevdokimov Moscow State University of Medicine and Dentistry, 20 Delegatskaya St., Moscow, 127473, Russia

Hepatocellular carcinoma (HCC) is the second leading cause of death in oncological patients. The prognosis of the disease outcome depends directly on its timely detection. Currently, in the majority of countries, the diagnostic algorithm at the preclinical stage of tumor development includes determination of alpha-fetoprotein in combination with instrumental imaging techniques. This approach allows the detection of about 65–80% of liver tumors at an early stage (A according to the BCLC classification), whereas at a very early stage (0 according to the BCLC classification) only 32–50% of cases, the result which cannot be considered satisfactory. In this regard, the search for effective biomarkers of hepatocellular carcinoma is an important challenge that faces the world healthcare.

Advances in proteomics and genomics have led to the discovery of numerous promising markers which are now being clinically tested. Molecules of protein nature proposed as hepatocellular carcinoma tumor markers in different periods of time are described in this review. Comparative data on their effectiveness and specificity are also presented. The possibility of isolated or combined use of these biomarkers for risk assessment and early diagnosis of primary liver cancer is considered.

Key words: hepatocellular carcinoma; screening; biomarkers; proteomics.

Introduction

Annually, about 700,000 diseases of hepatocellular carcinoma (HCC) are registered worldwide [1, 2]. Prevalence of liver cancer stands fifth among oncological diseases [3, 4] and in recent years, HCC moved from the third to the second place among the causes of death of oncological patients [5–8]. HCC-related morbidity rate (per 100,000 population) varies in different countries from 1.7 in Northern Europe and Canada to 30.0 in China where more than half of all cases of liver cancer is registered [9–11]. In Russia, 6–8 thousand new HCC cases are registered every year (4.0–5.0 cases per 100,000 population) which corresponds to an average rate of morbidity [12]. Worldwide, HCC occurs 3 times more often in men than in women [9, 13, 14]. Besides, there are substantial differences in HCC incidence in the representatives of various races: Mongoloids suffer from the disease 2 times more often than Negroids, while the Hispanics 2 times more often than white Americans [13].

Hepatocellular carcinoma develops more commonly in patients with liver cirrhosis (LC) caused by hepatitis viruses B and C, chronic alcohol abuse, and non-alcoholic fatty liver disease [12, 15, 16].

Since there are no clinical symptoms at an early stage of the disease, HCC is diagnosed late in 60% of patients and often concurrently with multiorgan metastasizing [1, 3]. If a tumor is detected at an early stage, the prognosis
is relatively good and 5-year survival exceeds 70% [5, 17–21].

All the above-said dictates the necessity of conducting active investigations and finding serum HCC biomarkers which are cost-effective and available for a wide screening [15, 22, 23].

Special attention is paid to the search for HCC biomarkers which would allow the tumors to be diagnosed with a high degree of probability at early stages when instrumental methods are yet ineffective [24, 25]. It is useless to apply oncomarkers at the advanced stage as ultrasound examination, computed tomography, and magnetic resonance imaging show high diagnostic sensitivity (Se) [26, 27].

Practical application of biomarkers is connected with the degree of their clinical trial. Five phases of biomarker validation are distinguished and their stepwise implementation provides the possibility to make an objective conclusion about their effectiveness (Table 1) [5, 28, 29].

At present, a great variety of biomarkers are declared at the preclinical stage. But though their possible usage is theoretically grounded and some limited clinical observations have been obtained, wide multicenter studies proving their actual value are absent [3].

The search for HCC biomarkers is being carried out in three principal directions: examination of protein markers (proteomics), nucleic acids and their polymorphisms (genomics), and determination of metabolites in blood and urine (metabolomics) [5, 19, 29–34]. In this work, information about protein oncomarkers, which are now of special interest, is overviewed. This is caused by the fact that methods of various protein indication are rather well automated, have high Se and reproducibility. Application of genomic oncomarkers in clinical practice is less common, the results of their identification vary greatly depending on the way of nucleic acid isolation, biological substrate, indication method. Serum and urine metabolites in cancerogenesis require further investigation, e.g. determination of a cut-off level for some specific metabolite typical for oncological disease. In other words, questions of specificity (Sp) in metabolomics acquire special importance when a diagnostic value of an oncomarker is concerned. Currently, canavanine succinate, glycochenodeoxycholic acid, and other organic acids, including fatty acids, are being studied as metabolomic indicators of cancerogenesis in liver tissues [35].

At early stages of liver cancer (0–A stages according to BCLC and stage I according to TNM classification), the diagnostic effectiveness of using different biological markers varies widely (Table 2).

### Table 1

| Phase | Stage of investigation | Purpose |
|-------|------------------------|---------|
| I     | Preclinical testing    | Search for candidate markers and theoretical grounding of their application |
| II    | Clinical analysis and validation | Testing for the possibility of using in clinical practice |
| III   | Retrospective analysis | Assessment of the effectiveness of using a biomarker according to the data of the retrospective studies |
| IV    | Prospective analysis   | Assessment of the effectiveness of using a biomarker at various stages of hepatocellular carcinoma and with consideration of false result frequency according to the data of the prospective studies |
| V     | Assessment of the effectiveness of diagnostic application in actual clinical practice | Analysis of the results of implementing a biomarker into clinical practice and their impact on the reduction of the hepatocellular carcinoma prevalence and morbidity in different countries and various ethnic groups |

### Table 2

| Biomarker         | The author suggested it as a HCC biomarker | Threshold value (cut-off) | Sensitivity (%) | Specificity (%) | References |
|-------------------|-------------------------------------------|---------------------------|----------------|----------------|------------|
| Alpha-fetoprotein (AFP) | Iu.S. Tatarinov, 1964 G.I. Abelev, 1968 | 10.9–400.0 ng/ml | 45.0 (24.0–66.0) | 88.0 (76.0–100.0) | [17, 19, 36–42] |
| Des-gamma carboxyprothrombin (DCP, PIVKA-II) | H.A. Lieberman et al., 1984 | 7.5–10.0 ng/ml | 40.0–200.0 mAU/ml | 46.0 (15.0–77.0) | 89.5 (81.0–98.0) | [14, 17, 43–48] |
| Glycosylated L3 isoform of alpha-fetoprotein (AFP-L3) | K. Taketa et al., 1990 | 5.0–15.0% | 36.5 (28.0–45.0) | 93.5 (90.0–97.0) | [17, 49–52] |
| Alpha-L-fucoidase (AFU) | M. Giardina et al., 1992 | 870.0 nmol/L | 81.9 (81.7–82.0) | 70.5 (70.0–71.0) | [39, 53–56] |
**Alpha-fetoprotein (AFP).** In spite of the fact that AFP has already been used in clinical practice for over 50 years, this biomarker remains most widely used to predict the development and monitor the efficacy of HCC treatment [5, 31, 39, 40]. AFP represents a glycoprotein with a 70 kDa molecular mass synthetized by endodermal cells of the fetal yolk sac and later by the embryonic hepatocytes [86].

Increase of the AFP level in the blood is observed in degenerative processes in the liver tissues and in various oncological diseases. The analysis of the literature assessing AFP as a HCC biomarker in patients with LC showed the range of Se and Sp from 41 to 65% and from 80 to 94%, respectively [96, 97]. Monitoring of the AFP level may be used to predict HCC recurrence after the operative treatment [98]. Data of AFP and US findings taken separately show Se to be below 50% at an early HCC stage but their combined application improves Se to 65% [23, 38].

In the regions with a high HCC prevalence, application of serological screening alone is permissible and justified [99]. There has been described a successful experience of HCC screening in patients with chronic hepatitis B in Alaska when US examination was impossible [100]. As a result, HCC was detected in part of the patients at the operable stage and early medical aid was provided [100].

Alpha-fetoprotein is a specific marker not only for HCC, its level rises in LC and tumors of the lungs, biliary tract, stomach, and pancreas [13, 39, 98]. Due to a low Se, the recent versions of European and American clinical recommendations excluded AFP identification from the diagnostic algorithm [19, 26, 40].

**Glycosylated L3 isoform of alpha-fetoprotein (AFP-L3).** AFP exists in three glycosylated isoforms [5, 7], and each possesses different capability to bind with lectin (lens culinaris agglutinin, LCA): AFP-L1 (non-LCA-bound fraction), AFP-L2 (weakly bound fraction), and AFP-L3 (LCA-bound fraction). AFP-L1 increases in chronic hepatitis and LC, whereas the elevation of AFP-L3 level is noted in tumor processes in the liver [17, 86]. Predominance of AFP-L3 in the total AFP level by more than 10–15% allows HCC to be suspected at an early stage of its development. A large multicenter prospective study [101] showed that Sp of AFP-L3 is approximately 92%, whereas Se is only about 37% irrespectively of the HCC stage.

A wide use of this biomarker is limited in clinical practice because the separation of the total AFP into fractions is possible only if its level exceeds 30 ng/ml [99]. Therefore, tumors producing no AFP cannot be detected by this method [23]. To overcome this disadvantage, a highly sensitive assay (hs-AFP-L3) has been developed in Japan which is applicable at low values of total AFP in the blood [82].

**Des-gamma carboxyprothrombin (DCP, PIVKA-II).** DCP is an abnormal form of prothrombin which is expressed due to the defect of post-translational carboxylation with underlying vitamin K deficit [102]. The other name of this oncomarker is a protein induced by...
vitamin K absence (PIVKA II). Functionally, DCP is a pathological inactive prothrombin [103]. Application of this biomarker with a diagnostic purpose showed good results in Eastern Asia, North America, China [104–106]. In Europe, the results were more controversial as its level was established to depend on the race and etiology of HCC [17, 48, 105, 106].

Des-gamma carboxyprothrombin may be used to assess not only the risk of HCC development but to predict its recurrence after surgical treatment. In the prospective study [107], application of DCP with AFP made it possible to suspect HCC 2 years before the diagnose verification. Further investigations are necessary in order to evaluate the effectiveness of this marker combination in the diagnosis of HCC [44]. It should be also kept in mind that DCP was mainly studied in Asian countries while the experience of using it in Europe remains insufficient.

**Alpha-L-fucosidase (AFU).** AFU is a lysosomal enzyme which breaks down glycoconjugates containing a carbohydrate — fructose [54]. Its activity has been shown to be higher in patients with HCC than with chronic hepatitis and healthy individuals [56, 108]. Application of AFU for screening purposes is not perspective due to a low Sp [109]. A high activity of this enzyme is found not only in HCC patients but in people with diabetes, pancreatitis, hypothyroidism as well [53, 109]. Besides, an average level of the enzyme activity depends on the person’s race and ethnic predisposition [53]. The AFU level has been reported to elevate in 85% of patients who 6 months later were diagnosed HCC on the basis of US findings [13, 110]. In its combination with AFP, Se increased to 95% and Sp to 99% during examination of HCC patients including those with advanced stage IV [55, 56].

**Glypican-3 (GPC3).** GPC3 belongs to a family of glypicans-proteoglycans. GPC3 is bound to the cellular membrane with a glycosyl-phosphatidyl-inositol anchor [3, 63]. GPC3 interacts with some growth factors [22, 63, 86] participating in cell proliferation and inhibition of cancer cell growth which characterizes it as an oncosuppressor [62, 63, 86]. An elevated GPC level is revealed in 50–55% of HCC patients [61] and only in 5% of those with LC [22, 63, 111]. At an early HCC stage (0 and A according to BCLC or I according to TNM), Se and Sp of serum GPC3 identification were 55.1% (47.9–66.2%) and 97.0% (95.2–98.2%), respectively [60].

An independent significance of the GPC3 for HCC diagnosis is restricted due to low Se [61, 86]. GPC3 is increased in 1/3 of patients with normal AFP indices in the blood [64]. Absence of correlation between these biomarkers gives grounds for their combined use [59, 77], improving Se to 76% for the tumors below 3 cm in size [64, 112]. GPC3 is determined immunohistochemically in liver biotapes which are used in clinics for differential diagnosis of HCC and other liver damages [13, 113].

**Golgi protein 73 (GP73).** GP73 is a specific membrane protein of Golgi complex which is usually expressed in the epithelial cells of different organs [114]. In the liver, it is synthesized mainly in the epithelium of the biliary tracts but when an inflammatory process develops its level in hepatocytes rises sharply [38, 86, 114, 115]. HCC is also accompanied by GP73 increase in the blood [13, 54, 86, 116]. GP73 is more sensitive than AFP and the elevation of its level concurrently with HCC formation begins earlier [67, 70]. GP73 level does not depend on the etiological causes of tumor development, the stage of cancerogenesis, or functional state of the liver, however, it, like many other biomarkers, does not show high Sp. GP73 rises in other liver tumors as well including cholangiocarcinoma [67, 114]. Another drawback of practical application of this biomarker is a considerable inaccuracy in identification of this protein at low concentrations in the blood [54]. If combined with other markers, e.g. with AFP and AFP-L3, the diagnostic value of GP73 increases [87, 116, 117].

**Squamous carcinoma antigen (SCCA) and immune complex SCCA-IgM.** SCCA antigen is a member of the family of high-molecular serine protease inhibitors which is present in squamous epithelium [5, 72, 86]. SCCA is synthesized in a large amount in epithelial tumors including cervix cancer cells. Giannelli et al. [69] have found a higher SCCA level in HCC patients relative to those suffering from LC. The identification of SCCA showed high Se but low Sp [71, 72]. The possibility of detecting SCCA in histological sections of tumor biotapes allowed it to be recommended as an immunohistochemical marker [118]. Guido et al. [119] have found that SCCA expression in neoplastic nodes is much higher than in regenerative ones.

The application of the immune complex SCCA-IgM rather than SCCA itself became an alternative variant. This complex is present normally in the desquamating epithelium and is not determined in the blood [54]. SCCA detection in the structure of the immune complex turned out to be more effective than identification of a free antigen [71, 119]. The frequency of SCCA-IgM identification in patients with chronic hepatitis, LC, and HCC was 18, 26, and 70%, respectively [54, 120] but Sp remained sufficiently low (50%) [71]. Evidently, these HCC markers will not have an independent value but their application in future as supplementary indicators in combination with some highly specific component is not excluded [72, 118].

**Osteopontin (OPN).** OPN, also known as transformed protein phosphatase (SPP1), represents an integrin-binding glycoprophosphoprotein which is produced in increased amount in many types of malignant neoplasms including cancer of the lungs, breast, intestine, pancreas, kidneys, gallbladder, prostate, ovaries [121–123]. In the physiological state, OPN is synthetized in the epithelium of biliary ducts, Kupfer cells but is not expressed in hepatocytes [35]. In 1999, an increased OPN production was found in the focus of hepatocyte necrosis caused by carbon
tetrachloride. Later, the elevated OPN was reported in many diseases of the liver such as viral hepatitis, acute liver insufficiency, non-alcoholic and alcoholic fatty liver disease [13, 76, 123]. Its key role has been suggested in the induction of inflammation and fibrosis of the liver. Thus, spontaneous liver fibrosis develops during a year in transgenic mice with OPN hyperproduction [123, 124]. Kim et al. [74] were among the first to assess the value of OPN in HCC. The OPN level rises long before (from 6 months to several years) the episode of the instrumental detection of HCC and has better Se than AFP [121, 125].

There is no correlation between the content of OPN and AFP in HCC patients, therefore, their combination is considered as a variant of an effective predictor of HCC risk development [41, 122, 126].

**Annexin A2 (Ann A2).** Ann A2 is a calcium-dependent protein binding phospholipids [19, 127]. It is expressed in the cells of different tissue tumors and plays a role in realization of the following processes: angiogenesis, proliferation, apoptosis, adhesion, invasion, cell migration [19, 128–131]. Its level changes in the variety of cancers such as cancer of the intestine, lungs, stomach, breast, esophagus [19, 33, 80]. Sun et al. [79] have observed the elevation of Ann A2 level in 83.2% cases in patients with HCC at 0 and A stages (BCLC classification) though Sp of this marker turned out to be low and did not exceed 67.5%. A combination of Ann A2 and AFP slightly improved Se but did not influence Sp (87.4 and 68.3%, respectively) [79]. In the HCC cohort producing no AFP, the Ann A2 level was elevated in 78.4% of cases. In this regard, Ann A2 is considered as a candidate biomarker for early HCC detection in patients with normal AFP indices in the blood serum [5, 33, 79, 80].

**Dickkopf-related protein 1 (DKK-1).** This is a secreted glycoprotein, inhibitor of intracellular beta-catenin signaling pathway [132, 133]. It participates in cell proliferation, differentiation, and apoptosis [3]. Increase of its content in the blood was found in oncological processes of various localization: cancer of the prostate, skin, liver [86, 134]. It shows a low Se level (65%) during HCC diagnosing but acceptable Sp (94%). Therefore, at early stages of HCC, identification of DKK1 is more effective than AFP [13, 46, 87, 133]. A combined use of AFP, DKK1, and DCP has been reported [87]. It increases Se and, consequently, the effectiveness of diagnosis of small-sized HCC [77, 87, 134].

**Receptor tyrosine kinase sAxl (AXL).** AXL is a tyrosine kinase which is involved in proliferation processes. It is expressed in many types of cells and its biological effects depend on the tissue specificity of the cells. An AXL form circulating in the blood has a molecular mass of 80 kDa and can be detected by the standard diagnostic methods [135, 136]. Functionally, AXL makes cancer cells resistant to chemopreparations [137–139]. Its increased production is observed in oncological diseases and correlates with poor prognosis [19, 89, 138–140]. In a large multicenter study, a diagnostic value of AXL for early HCC detection has been determined. AXL Se was found to be much higher than that of AFP [88]. A combination of AXL with AFP enables the improvement of Se method and preservation of Sp level within 90% [88].

**Heparin-binding growth factor midkine (MDK).** MDK has a low molecular mass. In the physiological state, the highest MDK level is observed in the midgestation period which reflects the name of the protein, *midkine* (the middle of kinetics). The MDK level in the serum of healthy people does not usually exceed 0.5–0.6 ng/ml while in patients with malignant diseases it is substantially higher [141]. There has been described an elevated MDK expression in tumors of different tissues and organs such as neuroblastoma, glioblastoma, cancer of the thyroid gland, lungs, esophagus, stomach, and prostate [141, 142]. In the investigation carried out on patients with HCC and LC, it has been established that MDK levels in the first cohort of patients were on average 5 times higher than in the second. The calculated Se for MDK was 90% and for AFP only 50% [141, 142].

**Minichromosome maintenance protein 6 (MCM6).** This is a component of a protein complex maintaining minichromosome. It takes part in the process of DNA replication during the S-phase of the cell cycle [143]. In a single work [93], a promising outlook of MCM6 application has been shown for early detection of HCC at a satisfactory Se level. However, the cohort of the examined patients in this study differed by clinical criteria from the cohort indicated in the BCLC classification, therefore the value of MCM6 for diagnosis of early and very early HCC stages remains unclear [93, 144, 145].

**Thioredoxin (TRX).** TRX represents thioredoxin which reduces disulfide bonds of the proteins. TRX is involved in the biological processes such as regulation of protein synthesis, apoptosis, and cell proliferation and provides protection against oxidative stress [146]. TRX expression is elevated in the tumor cells and its level correlates with the prognosis which has been shown in cancer of the lungs and colorectum [147, 148]. Li et al. [94] have reported the potential possibility of using TRX for early revealing of HCC. In this study, TRX Se and Sp (74.9 and 87.5%, respectively) were higher than for AFP (68.6 and 75.2%, respectively). A combination of TRX and AFP raises the effectiveness of HCC diagnosis (Se — 81.3%; Sp — 93.4%; AUC — 0.889), therefore, their joint application is justified but requires more ample study [94, 149].

**Soluble urokinase plasminogen activator receptor (suPAR).** SuPAR is a circulating form of the membrane protein — a receptor activator of urokinase-type plasminogen [95]. In 2015, suPAR was used to assess tumor progression and cancer metastasizing [79]. The suPAR level in the serum is elevated in patients suffering from cancer of the ovaries, colon, and HCC [79, 95]. In the prospective study [95], the occurrence of HCC in 267 patients with chronic hepatitis has been investigated.
for 7 years. This work showed the possibility of using this marker for HCC prediction at Se 76.0% and Sp 90.4%. These results lead to the conclusion that suPAR possesses a certain potential as an early predictor of the risk of HCC development.

Conclusion

The search for hepatocellular carcinoma biomarkers on the basis of proteomics is intensively being carried out in the countries worldwide since the detection of this disease at its early stage substantially influences the results of operative treatment and patients’ quality of life. An ideal biomarker of hepatocellular carcinoma must be applicable for screening and reach the level of Se and Sp exceeding 90%. Besides, the methodology of investigation should be minimally invasive and economically grounded [5].

In order to reach this goal, the majority of current strategies make provision for the use of new molecules in combination with previously discovered [5, 19, 29]. It is important that these markers are not associated with each other and do not give diagnostic cross results [5, 31]. Additionally, certain diagnostic algorithms and mathematical formulas are proposed for use combining quantitative results of identification of 2–4 markers and biochemical tests [33, 52, 56, 87, 102, 141]. The diagnostic value of different algorithms and diagnostic scales continue to be assessed in the prospective clinical studies being performed [150–152]. Despite a positive experience of using this approach in Japan, China, Korea, and other countries with a high morbidity from hepatocellular carcinoma, the main limiting factor of its implementation in clinical practice has been until now the cost of the analysis which grows significantly when a combination of markers and the corresponding diagnostic algorithms pertaining to them are applied [153].

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