

Present and future possibilities for early diagnosis of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) represents the fifth most common cancer in the world, and the third most frequent oncological cause of death. The incidence of HCC is on the increase. HCC typically develops in patients with chronic liver diseases, and cirrhosis, usually with viral etiology, is the strongest predisposing factor. Nowadays HCC diagnosis is a multistage process including clinical, laboratory, imaging and pathological examinations. The prognosis of HCC is mostly poor, because of detection at an advanced, non-resectable stage. Potentially curative treatment (surgery) is limited and really possible only for cases with small HCC malignancies. For this reason, more effective surveillance strategies should be used to screen for early occurrence of HCC targeted to the population at risk. So far, the generally accepted serological marker is α -fetoprotein (AFP). Its diagnostic accuracy is unsatisfactory and questionable because of low sensitivity, therefore there is a strong demand by clinicians for new HCC-specific biomarkers. In this review, we will focus on other biomarkers that seem to improve HCC diagnosis, such as AFP-L3, des- γ -carboxyprothrombin, α -L-fucosidase,

γ -glutamyl transferase, glypican-3, squamous cell carcinoma antigen, a new generation of immunoglobulin M-immunocomplexes, and very promising gene-expression profiling.

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Key words: Hepatocellular carcinoma; Chronic hepatitis; Liver cirrhosis; Cancer screening; Surveillance; Biological markers

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major challenge in contemporary medicine. The incidence of HCC is on the increase and it is becoming more and more significant both clinically and epidemiologically. Now HCC represents the fifth most common cancer in the world and the third most frequent cause of mortality amongst oncological patients^[1]. It is responsible for more than 500 000 deaths with over 600 000 new cases yearly worldwide^[2]. Incidence rates are different in various countries: highest in South-East Asia and Sub-Saharan Africa (around 120/100 000) and lowest in the USA (1.8/100 000) and Western Europe (3-5/100 000)^[1,2]. Although Poland belongs to the group of countries with a relatively small incidence rate: lower than 6/100 000 in men and 3/100 000 in women, HCC causes the death of more than 2500 Poles every year and, according to statistically observed trends, the mortality rate will gradually increase^[3].

More than 95% of HCC patients present underlying hepatopathy - in particular of viral etiology (Table 1)^[4]. The majority of the cases (> 85%) have liver cirrhosis, which masks symptoms of cancer progression. The clinical course of HCC is mostly asymptomatic. Suspected focal liver changes are often detected incidentally while monitoring the patient's condition during abdominal ultrasound (US) examination, and often are too large and too advanced for the tumor to be subjected to potentially effective and radical therapy.

In 1999 in "Hepatology", Llovet *et al*^[5] published the results of an analysis of clinical data of 102 patients with unresectable HCC. They found that 80% of patients with asymptomatic unresectable HCC survived for 1 year, 65% for 2 years, and 50% for 3 years. Only 29% of patients with clinical symptoms who did not have radical therapy survived for 1 year, 16% for 2 years, and 8% for 3 years^[5]. Despite great medical progress since the times of Llovet's report and huge developments in medicine, patients suffering from HCC presently cannot be offered much more. Because of serious limitations of the surgical and oncological treatment available, it seems necessary to concentrate on the earliest possible diagnosis, particularly sensitive detection of resectable focal liver changes - preferably when tumors are less than 2 cm in diameter^[6]. For this reason, surveillance with US techniques and serum α -fetoprotein (AFP) analyses is recommended for all cirrhotic patients and other specific risk groups (Table 2)^[7] every 6 mo.

RADIOLOGICAL TECHNIQUES FOR HCC DIAGNOSIS

US is the most popular method for HCC screening. Diagnostic success of US for HCC surveillance depends on many factors, but mostly on the size and character of the focal liver changes, as well as the experience of the sonographer and the technical quality of the US equipment. According to the literature^[8,9] US sensitivity rises from 70% for lesions of about 1 cm in diameter, towards 90% when the tumor diameter is more than 5 cm. The specificity is variable between 48% and 94%^[8,9]. HCC does not have a specific morphology on US, whereas smaller lesions, less than 3 cm in diameter, are homogenic and hypoechoic. As they increase and form focal necrosis and microbleeding, they become more and more heterogenic and hyperechoic. This feature together with arterial vascularity are typical of increased malignancy and poor prognosis. Doppler or contrast-enhanced US leading to a better visualization of the relation between organic neoplasms and vascular structures may be used for clear differentiation of those lesions. Because US examination is subjective and non-repetitive, all focal liver lesions suspected on US should be verified using: computer tomography (CT) and/or magnetic resonance imaging (MRI). The use of these methods leads to a much more accurate diagnosis of HCC: sensitivity up to 89% and specificity reaching 99%^[9]. Unfortunately, the diagnosis seems not to be so precise when

Table 1 HCC risk development factors (%)^[4]

	Europe	North America	Asia & Africa	Japan
HCV	60-70	50-60	20	70
HBV	10-15	20	70	10-20
ALC	20	20	10	10
Other	10	10	0	0

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ALC: Alcohol abuse.

Table 2 HCC specific risk groups (AASLD Practice Guideline 2005)^[7]

Hepatitis B carriers	Non-hepatitis B carriers
Cirrhotic patients (incidence of HCC: 3%-5%/year)	Hepatitis C (incidence of HCC: 2%-8%/year)
Non-cirrhotic patients with high HBV DNA and hepatitis activity	Alcoholic cirrhosis
Positive family history of HCC	Genetic hemochromatosis
Asian males > 40 years (incidence of HCC: 0.4%-0.6%/year)	Primary biliary cirrhosis and autoimmune hepatitis
Asian females > 50 years (incidence of HCC: 0.2%/year)	α 1-antitrypsin deficiency
Africans > 20 years (incidence of HCC: > 0.2%/year)	Non-alcoholic steatohepatitis

lesions are less than 1 cm in diameter - merely 34%^[10].

Many epidemiological studies showed that only 50% of HCC lesions smaller than 1 cm in diameter are discovered during US examination. According to the Barcelona recommendations those lesions should be observed, or, more precisely, screened by US at a minimum of 6 mo intervals. When the tumor is growing and/or becomes larger than 1 cm in diameter on US, CT and/or MRI should be applied. It is recommended that focal liver changes > 1 cm but < 2 cm be subjected to histopathological verification. False negative results have been found in 40% of patients subjected to a targeted liver biopsy^[11], therefore exclusion of HCC in this way seems meaningless. If a tumor is > 2 cm in diameter with pathognomic arterial hypervascularity verified by other radiological methods, and there is a high level of total AFP serum concentration (> 400 ng/mL), then HCC can be diagnosed, according to the Barcelona criteria^[12,13].

At present more and more doubts have been raised about using AFP as a reliable HCC biomarker. For this reason, American hepatopathologists treat every tumor larger than 1 cm in diameter in cirrhotic liver as HCC, consequently ignoring AFP serology (very frequently false negative)^[14].

HCC SPECIFIC BIOMARKERS

AFP

AFP was discovered in 1956 by Bergstrand and Czar^[15], who used paper for electrophoretic separation of human fetoprotein in serum. The first reports on the usefulness of AFP as a diagnostic marker for HCC were presented

Table 3 Diagnostic values of AFP as HCC biomarker^[20,21]

Cut-off value (µg/L)	Sensitivity (%)	Specificity (%)	Ref.
20	55-60	88-90	[21,22]
50	47.0	96.0	[22]
100	31.2	98.8	[21]
200	22.4	99.4	[21]
400	17.1	99.4	[21]

AFP: α-fetoprotein.

in 1964 by Tatarinov and in 1968 by Abelev^[16]. AFP is a glycoprotein with molecular weight of around 70 kDa, synthesized in the endodermal cells of the yolk sac during early fetal development, and then in embryonic hepatocytes^[17]. It reaches a maximum serum concentration of 3 g/L in the 12-16th wk of fetal life and during the next 18 mo, AFP values decrease and normalize^[18]. Its synthesis in adult life is repressed. Pathological elevation is seen in hepatocyte regeneration and hepatocarcinogenesis. Numerous data have proved that significantly higher AFP serum levels accompany various liver diseases (viral hepatitis, liver cirrhosis, liver tumors: primarily HCC and hepatoblastoma, also metastasis in 5%-10% cases), other neoplasms (mainly cancers of the digestive tract: pancreas -24%, stomach -15%, large intestine -3%, and gallbladder). Positive predictive values (PPV) for AFP are paradoxically significantly lower among patients with HCC viral etiology than non-viral (PPV: 70% vs 94%, $P < 0.05$)^[19]. It has been confirmed on numerous occasions that AFP serum concentration increases in parallel with HCC tumor size. For this reason AFP has to be considered 'the golden standard' for HCC serum markers. However, the usefulness of AFP testing for the population at risk should be seriously questioned. AFP diagnostic values for this assay are undoubtedly poor. AFP specificity varies from about 76% to 96% and increases with elevated cut-off value. Simultaneous sensitivity decreases much more from about 25% for potentially resectable tumors of less than 3 cm in diameter to about 50% for lesions of > 3 cm in diameter (Table 3)^[20,21]. 20%-30% AFP sensitivity coincides with cut-off values > 100 µg/L, which means that 70%-80% of the results - of a conventional test used as "a gold standard" for HCC diagnosis - are falsely negative. Based on these data 70%-80% of liver tumors, normally resectable are non-detectable. For this reason only one patient out of 5 receives potentially curative treatment. Unfortunately, the remaining majority unfortunately do not undergo treatment or are subjected to it too late.

Nowadays it is believed that early HCC diagnosis is presently considered feasible in 30%-60% of the cases in developed countries. Tumors smaller than 2 cm in diameter represented < 5% of cases in the 1990s in Europe, whereas now they represent up to 30% of cases in Japan^[6]. Significantly more effective surveillance strategies in Japan lead to earlier HCC detection and earlier qualification for effectively curative radical surgery, with very good postoperative survival rates^[22]. According to these assumptions, European and American experts have defined trends and

expected aims of surveillance policies in Western countries in 1980-2020. The applicability of potentially curative treatments have been divided into 3 periods: until 1990: 5%-10% of cases; 1990-2010: 30%-40% of cases; and 2010-2020: 40%-60% of cases^[6]. Limitations of available therapies constitute a major challenge for diagnostic techniques, which are, most of all, modern visual methods and novel HCC specific biomarkers.

Numerous studies analyzing the chemical structure of AFP have shown that different sugar moieties of the bonds determine their binding capacity to lectin lens culinaris agglutinin (LCA)^[23]. Taking those facts into consideration, Polish scientists, Breborowicz *et al*^[24] identified in 1981 3 main glycoforms, namely AFP-L1, AFP-L2, AFP-L3. AFP-L1, the non-LCA-bound fraction, is the major AFP isoform in the serum of nonmalignant hepatopathy patients (chronic hepatitis, cirrhosis). AFP-L2 presents intermediate binding capability with its serum concentration increasing during pregnancy, and it is also present in cases of yolk sac tumors. AFP-L3, as the LCA-bound fraction, is the major glycoform in the serum of HCC patients^[24-26]. It can be detected in 35% of patients with small HCC (< 3 cm). Some clinical studies have indicated that AFP-L3 can be detected 9-12 mo ahead of changes using visual techniques^[27,28]. Sensitivities of AFP-L3 in detecting HCC range from 45% for lesions < 2 cm to > 90% for changes > 5 cm in diameter^[20]. The specificity is more than 95%^[26,29]. Fucosylation rate can be used in clinical practice (AFP-L3/AFP total). It has been confirmed that the ratio of more than 10% is closely associated with worse liver function and poorer tumor histology with implications such as larger tumor mass, a more invasive/malignant character, and earlier metastatic tendency^[27,28]. Therefore AFP-L3 could be used as a reliable early HCC biomarker and a valuable indicator of poor prognosis. It is possible to achieve particularly accurate results for HCC screening with the use of AFP-L3 in combination with one of the 3 newly-discovered AFP glycoforms which can also be used as single tests, that is AFP-P4, AFP-P5 (E-PHA), and monosialylated AFP (IEF)^[19].

Des-γ-carboxyprothrombin (DCP)

DCP, also known as PIVKA-II (protein induced by vitamin K absence or antagonist-II), is an abnormal, inactive prothrombin, lacking carboxylation of the 10 glutamic acid residues in the N-terminus, which is the result of an acquired post-translational defect of the prothrombin precursor in HCC cell lines. DCP was discovered in serum of patients during their anticoagulant therapy with a vitamin K antagonist. In 1984 Liebman *et al*^[27] first described a higher DCP level both in patients with HCC and in cases of HCC recurrence after surgical resection, suggesting the usefulness of DCP as an HCC biomarker. It has been proved that significant concentrations of serum DCP are present in 50%-60% of all HCC patients, but in only 15%-30% of early HCC cases^[31]. In the analyses of Nakagawa *et al*^[32] the sensitivity of this test is 48%-62% and the specificity is 81%-98%. The diagnostic value of DCP as a biomarker is

Table 4 Diagnostic values of HCC serum markers^[34-36]

Type of test	Sensitivity (%)	Specificity (%)
AFP-L3	61.60 ^[34]	92.00 ^[35]
DCP	72.70 ^[34]	90.00 ^[35]
AFP	67.70 ^[34]	71.00 ^[35]
AFP-L3+DCP	84.80 ^[34]	97.80 ^[37]
AFP-L3+AFP	73.70 ^[34]	86.60 ^[35,36]
DCP+AFP	84.80 ^[34]	90.20 ^[35]
AFP-L3+DCP+AFP	85.90 ^[34]	59.00 ^[35]

DCP: Des- γ -carboxyprothrombin.

approximately comparable with AFP. Grazi *et al*^[33] proved that AFP and DCP are not correlated, so the combination of those markers significantly improves HCC detection: sensitivity 74.2%, specificity 87.2%. Carr *et al*^[34] reported in 2007 interesting data based on prospective analyses of 99 patients with non-resectable HCC verified using liver biopsy (Table 4)^[34-36]. Nowadays the best way to diagnose HCC is the use of AFP-L3 with DCP analyzed by immuno-enzymatic higher sensitivity methodology^[33,37].

α -L-fucosidase (AFU)

AFU is a normal lysosomal enzyme which hydrolyzes sugars containing L-fucose. In 1984 Deugnier *et al*^[38] first reported that AFU is overexpressed in patients with HCC liver changes. It has been proved that the values of AFU serum concentration were not correlated with the tumor size and were frequent in early HCC cases^[23]. Tangkijvanich *et al*^[39] indicated that the sensitivity and specificity of AFU were about 80% and 70% respectively, in contrast with 40% and almost 100% for AFP. A simultaneous determination of both markers can improve the sensitivity to 82%^[39]. This conclusion suggested that AFU could serve as a valuable supplement to AFP in early detection of HCC, similar to another popular serum enzyme - γ -glutamyl transferase.

γ -glutamyl transferase (GGT)

GGT is a glycosylated membrane enzyme which activity is modulated in many physiological and pathological conditions, including differentiation and carcinogenesis^[40]. It is mainly secreted by the hepatic Kupffer cell and endothelium of the bile duct. GGT is also overexpressed, similar to AFP, by fetal hepatoblasts and HCC cell lines^[23]. The total serum GGT, a generally accepted cholestatic marker, has poor HCC specificity so can be useful only supplementary to AFP and other newer biomarkers for more effective HCC screening. In 1965, Polish scientists Kokot *et al*^[41] separated the serum γ -glutamyl transferase into 3 to 4 bands by means of paper electrophoresis^[38]. Since then, other methods have been used, that is, separation of GGT bands by means of starch gel (Orlowski *et al*^[42]), cellulose acetate (Hitoi *et al*^[43]), agarose gel (Hetland *et al*^[44]), polyacrylamide gel electrophoresis (Kojima *et al*^[45], Suzuki *et al*^[46], Sawabu *et al*^[47], Kew *et al*^[48]), and polyacrylamide stage gel plate (Xu *et al*^[49]). Xu reported that they had fractionated 9 to

11 activity bands of GGT, in which GGT II was found in the sera of all patients with hepatoma. The positive rate of GGT was 90% and no correlation was observed with AFP^[49] and DCP^[50]. After 10 years of follow-up they reported that GGT II was positive in 90% of cases with HCC and negative in most patients with acute and chronic viral hepatitis, extrahepatic tumors, in pregnant women, and in healthy controls^[51].

Glypican-3 (GPC-3)

GPC-3 is an oncofetal protein being one of the members of heparan sulfate proteoglycans anchored to the plasma membrane through glycosylphosphatidylinositol^[52]. GPC-3 is normally involved in the regulation of cell proliferation and survival during embryonic development and functions as a tumor suppressor. It has been reported to be downregulated in breast cancer, ovarian cancer and lung adenocarcinoma^[53] but upregulated in HCC^[54]. GPC-3 is absent in hepatocytes of healthy subjects and patients with nonmalignant hepatopathy, and can be detected in about 50% of HCC patients and 33% of HCC patients seronegative for both AFP and DCP. The specificity of GPC-3 is 100%^[55]. Some clinical studies have indicated that the simultaneous determination of GPC-3 and AFP could significantly increase the sensitivity in HCC detection, without a reduction in the specificity^[56]. More trials have confirmed the diagnostic value of 2 other, newly-discovered membranous proteins: Golgi protein 73 (GP73) and mucin 1 (MUC-1).

GP73 is a resident Golgi protein, shown to be upregulated in hepatocytes of patients with acute hepatitis^[57] and cirrhosis^[58] and in the sera of patients with HBV- and HCV-related HCC^[59,60]. Marrero *et al*^[60] reported a sensitivity of 69% and a specificity of 75% in HCC versus cirrhotic patients, indicating its superiority in comparison with AFP: sensitivity 30%, specificity 96%.

MUC-1 is a membrane protein expressed in many epithelial cells, but overexpressed in patients with breast cancer^[61], inflammatory lung diseases^[62], and HCC^[63,64]. Moriyama *et al*^[63] demonstrated expression of MUC-1 in HCC cells and in serum of patients with HCV-related HCC. Gad *et al*^[64] reported specificity of 99%, sensitivity of 87% for combined MUC-1, DCP and AFP in Japanese and Egyptian patients with HCC.

Squamous cell carcinoma antigen (SCCA)

SCCA represents a family of serine proteases of high molecular weight, also known as serpins. There are 2 homologous genes: *SCCA1* and *SCCA2*, encoding 2 different SCCA isoforms, both expressed in many normal squamous epithelial cells. Increased SCCA levels have been detected in head and neck cancers and other epithelial malignancies, including cervix and lung. Recently Pontisso *et al*^[65] first reported a high SCCA expression in HCC tissues, which seems very interesting, as liver does not possess squamous epithelial cells. Hepatocytes, however, share a common embryogenic origin. The sensitivity and specificity for SCCA in HCC diagnosis are 84% and 46% respectively. The complementary strengths

(high sensitivity/low specificity) and total AFP (low sensitivity/high specificity) suggest that the combination of the 2 markers should be of more value for screening, and in fact it leads to a diagnostic accuracy of 90%^[66].

Markers (AFP, SCCA, DCP) in immunocomplexes with immunoglobulins of the IgM class (AFP-IgM IC, SCCA-IgM IC, DCP-IgM IC)

A new step for HCC testing is represented by forming known antigens (AFP, SCCA, DCP) into immunocomplexes (IC) with immunoglobulins of the IgM class. Monitoring of SCCA-IgM IC, AFP-IgM IC and DCP-IgM IC appears to be a much more advantageous approach for detecting patients with small HCC changes^[67-70]. Giannelli *et al.*^[67] confirmed that the combined use of AFP IgM IC, SCCA and SCCA IgM IC in patients displaying low levels of AFP (< 20 IU/mL) identified 25.6% HCC. This study suggests that the use of a combination of all these markers in clinical practice provides a non-invasive and simple test that could increase the accuracy of HCC diagnosis. According to the results of Beneduce *et al.*^[68]: SCCA IgM IC significantly improves accuracy of HCC testing with sensitivity of 100%, specificity of 70%, PPV of 100%, and negative predictive value of 83%; AFP-IgM IC is a complementary serological marker to free AFP and the combination of these biomarkers may be useful in the diagnosis of liver cancer^[69]; DCP-IgM IC in HCC patients was not associated with an increase in IgM concentration and was more frequently detected in HCC patients than DCP and AFP, strengthening the diagnostic role of IgM immune complexes in liver cancer^[70]. The novel generation of HCC biomarkers seems very promising as it introduces new hope in supporting US for more accurate HCC screening.

CONCLUSION

The distinction between early HCC changes and dysplastic nodules among cirrhotic patients is challenging even in expert hands. It frequently proves very difficult to characterize by available radiological and pathological examination. Serum biomarkers such as AFP, AFP-L3, DCP, AFU, GGT, GP-73, MUC-1, SCCA, GPC-3 and a new generation of IgM-immunocomplexes have significant diagnostic limitations, and in fact they are not particularly precise for the early diagnosis of HCC. Simultaneous determination of these markers in various combinations could improve the accuracy in differentiating HCC from nonmalignant hepatopathy, but there still exists the unresolved problem of tiny 'grey' nodules in the 'black and white' diagnostic perspective. The potential of gene-expression profiling as a novel tool to improve diagnostic and prognostic prediction is very exciting. The development and progression of HCC is known to be caused by an accumulation of genetic changes resulting in an expression of cancer-related genes: oncogenes, tumor suppressor genes, genes involved in many regulatory pathways, such as cell cycle control, apoptosis and angiogenesis. Modern technology enables investigators to measure the expression of thousands

of mRNA's simultaneously and therefore may provide comprehensive information for diagnosis and therapy of HCC. Currently there are many defined lists of genes selected for the HCC molecular index such as telomerase reverse transcriptase, topoisomerase II α , heat shock protein 70, serin/threonine kinase 15), phospholipase A2, insulin-like growth factor 2, connexin 26, chemokine C-X-C motif ligand 12, α -2-macroglobulin, plasminogen, thrombospondin 1, and platelet-derived growth factor receptor α ^[71,72]. According to novel advancements in the management of HCC in 2008 by Llovet *et al.*^[73], high accuracy rates are presented by a 3-gene set: glypican-3, LYVE1 (lymphatic vessel endothelial hyaluronan receptor-1), and survivin. However, more studies are needed to demonstrate its superiority, and presently this is not the first choice in research on early detection of HCC. Major limiting factors for routine use of molecular technology in a clinical setting at present are the cost and the access to them. Hopefully in the not so distant future the costs will decrease and this technology will become increasingly more popular and automated.

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