Biomarkers for Hepatocellular Carcinoma (HCC): An Update

12

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Abstract

The past decades have witnessed increased use of biomarkers in disease management. A biomarker is any characteristic that can be objectively measured and evaluated as an indicator of normal biological process, pathogenic process, or pharmacological response to a therapeutic intervention. The clinical measurements of biomarkers can be carried out in vivo using imaging modalities like ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI), as well as in vitro utilizing serum or plasma or other body fluids as specimens. In contrast to the imaging modalities, a prominent value of serum biomarkers is that they could be biologically relevant and disease-specific to pathophysiologic or pathologic process of disease development. This article provides an update of serum biomarkers for hepatocellular carcinoma (HCC) in risk assessment for early detection through surveillance.

Keywords

AFP-L3 • AFP-L3 clinical performance • Alpha-fetoprotein isoforms • Chip-based microfluidic assay • Chronic hepatitis • Cirrhosis • DCP clinical performance • Des-gamma-carboxy prothrombin (DCP) • HCC • HCC biomarkers • HCC risk factors • Level of evidence • Liver fibrosis

12.1 Introduction: Some Important Issues Associated with HCC Early Detection Through Risk Assessment in Surveillance

The past decades have witnessed increased use of biomarkers in disease management. A biomarker is any characteristic that can be objectively measured and evaluated as an indicator of normal biological process, pathogenic process, or pharmacological response to a therapeutic intervention

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[1]. The clinical measurements of biomarkers can be carried out in vivo using imaging modalities like ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) as well as in vitro utilizing serum or plasma or other body fluids as specimens. In contrast to the imaging modalities, a prominent value of serum biomarkers is they could be biologically relevant and disease-specific to pathophysiologic or pathologic process of disease development.

Alpha-fetoprotein (AFP) has been widely used although it has not been formally approved or cleared by the U.S. Food and Drug Administration (FDA) as a cancer biomarker for hepatocellular carcinoma (HCC). Recently the regulatory agency has cleared two novel and specific HCC serum biomarkers for risk assessment for HCC, Alpha-fetoprotein-L3 (AFP-L3) and Des-γ-decarboxyprothrombin (DCP). AFP-L3 is a glycosylation variant of the AFP [2]. DCP is abnormal coagulation protein produced in liver and a precursor of thrombin in the coagulation cascade [3]. This review article will focus on analytical and clinical validity of the AFP-L3 and DCP as serum biomarkers and provide an overview of their potential clinical utilities in HCC management especially for early detection through risk assessment. I critically review some recent clinical research data up to 2012. The discussions are mainly from clinical laboratory perspectives with focus on the new microfluidic chip-based assay system, *μTASWako™i30*. This article discusses some important investigator-initiated studies and reports reflecting user experiences which have enriched the knowledge base of the novel oncology biomarkers and their potentials in medical practice.

12.1.1 Natural History: Disease Spectrum and Cellular Heterogeneity

Natural history of malignant disease and its relation to disease development is increasingly delineated and defined at the molecular levels [4], which offer ample opportunities and rich sources for biomarker assay developments. The disease development of HCC, like many other human malignancies, is not an event but a process which spans from physiological changes such as quantitative variations of biomolecules to pathological modifications of qualitative natures such somatic mutations [5].

Major risk factors in natural history of HCC have been understood thanks to intensive medical research on the viral etiology and mechanisms of the hepatitis B and C virus. Primary liver cancer is mostly HCC, the malignant disease of the hepatocytes [6]. Chronic hepatitis infections including hepatitis B in the Southeast Asia countries and hepatitis C in the West have been attributed to the rise in incidence of HCC over the past decades. In fact, the HCC has the fastest rising cancer incidence in the US [7]. Chronic hepatitis C infection will become cirrhotic within 20–30 years, however, as many as 40 % of chronic hepatitis B patients may not have clinical evidence of liver cirrhosis as a precursor to HCC [8].

HCC, also similar to most other human cancers, is heterogeneous [9, 10] which may be non-heritable in the sources of cellular diversity such as arising from different cancer stem cells [11] or heritable such as from driver mutations in different signal transduction pathways within the HCC cancer cell population [12]. Therefore depending on the driver mutation, HCC is probably not a single disease entity, instead is a collective term for many subgroups of the liver malignancies.

The early HCC is clinically manageable or curable [8]. The diagnosis for early intervention decision has shifted to relying more on non-invasive clinical diagnosis based on dynamic imaging modalities instead of histology in recent years [13]. In order to treat HCC more effectively, the size of the tumor nodule(s) when they are found by screening ideally should be single and less than 2–3 cm in diameter [14]. Given the background liver disease of cirrhosis, a liver nodule of <1 cm in diameter is rarely diagnosed as liver cancer [15]. A liver nodule, when detected, the size of <1–2 cm is considered to be in the early stages for the purpose of the discussion.

Serum biomarkers are useful assisting in the characterization of a liver nodule for evaluating likelihood of HCC occurrence, or its downstream

risk of evolving into HCC within a specific timeframe. Surveillance can improve survival by reducing deaths using US and serum biomarker [16–18]. In Japan, surveillance has been embedded into medical practice [19, 20]. Although clinical conditions limiting successful management of HCC exist, such as the residual liver functions among others in patients with the HCC, early detection of HCC do offer additional treatment options. It has been observed that patients with end stage liver diseases could have excellent long term survival if pre-matured deaths from HCC can be prevented through surveillance and liver transplantation [21, 22].

12.1.2 Serum Biomarkers: Specificity Versus Sensitivity

American Association for the Study of Liver Diseases (AASLD) has recommended that HCC surveillance should be based on US every 3–6 months for patients at high risk for HCC [15]. These patients are mainly chronic HBV carriers of Asian males over 40 years and female over 50 years old and cirrhotic HCV infection [15]. It is concluded that US is effective as the first line HCC surveillance [15]. However, some medical experts are quick to point out that US is limited by its relatively low sensitivity and therefore the high false negative rate for use in the HCC surveillance. US demonstrated marginal performance in clinical sensitivity for HCC surveillance with sensitivity of 60 % on average [23]. Furthermore, US is also operator-dependent with significant performance variations among hospitals and medical centers around the country. The US images are subject to human interpretations and are vulnerable to human errors. The effective use of US in surveillance has been hampered by poor reproducibility [24]. Other limiting factors for diagnostic grade of US results are physiological or pathological in nature such as fatty liver disease associated with metabolic syndrome, interference from the anatomical barriers adjacent to the liver such as lung or stomach that could sometime obscure the imaging producing less granular pictures. The background cirrhotic liver could also have potential cripple effect on US quality.

There should be no doubt that serum biomarkers could provide additional diagnostic or prognostic information. More information would likely change the clinical impression on likelihood of HCC especially in some challenging clinical diagnostic situations which more often than not is the rule rather than the exception in HCC management because of the background or underlying liver cirrhosis leading to the HCC development.

Serum biomarkers can signal the early development of HCC. In general, HCC is derived from liver cirrhosis presenting as background liver disease of high grade dysplastic nodules due to the chronic viral hepatitis infection [25]. Liver nodule(s) can be detected by US as mass(es) of sufficiently large size, say, for example when it reaches 2 cm in diameter or greater. Serum biomarkers can be an early warning alerting the development of HCC. Early diagnosis by surveillance is associated with lower mortality risk [18]. The HCC with seropositive AFP-L3 is reported to be correlated to short doubling time in tumor volume, and increased arterial supplies of tumor nodule, thereby clinically aggressive with poorer prognosis [26, 27]. Newer generation of the AFP-L3 assay is highly sensitive for pathologically advanced HCC [28]. It is worth noting that the pathologically advanced HCC may be more clinically aggressive even they are small in size for example <2 cm [26].

Clinically useful Serum biomarkers should have several key characteristics. They must be cancer specific, non-invasive and safe to use, convenient and easy to apply in different clinical settings, and acceptable to patients. They are expected being sensitive to the underlying disease. However, clinical sensitivity could be affected by a variety of analytical and biological reasons. Undesirable detection limit of assay technology could affect the clinical sensitivity. Improvement in assay's detection limit can increase the true sensitivity but also the false positive rate by decreasing the assay specificity simultaneously. Biologically, tumor heterogeneity could also curtail the sensitivity of a laboratory

assay because some cancer may not produce certain biomolecules as serum biomarkers especially in early stage. It was suggested this is also the case in HCC [29, 30]. There are different subgroups of HCC. For instance, approximately 20–80 % of the HCC do not have elevated AFP depending on tumor size at diagnosis [31]. This has been a significant issue when clinicians use AFP for referral of patients suspicious of HCC for imaging confirmation.

The clinical sensitivity can be significantly improved by advanced assay technology with drastic improvement in lower detection limit thereby higher analytical sensitivity. For example, the *i30* AFP-L3 and DCP assay platform based on microfluidic chip has greatly improved the analytical sensitivity [32]. But physicians are compelled to address the issue of "false positivity" of the test results. It is expected that the surveillance strategy for HCC management with multiple periodic sampling could in some degrees provide practical solution to the issue with respect to whether these seropositive AFP-L3 and DCP cases are authentic HCC in patients at high risk for the malignant liver disease.

The other reason for the low clinical sensitivity of some cancer biomarkers must be biological due largely to cancer is heterogeneous with many subgroups as demonstrated by recent studies in breast cancer [4]. It is clear also from molecular studies that HCC is likely not a single disease entity according to the underlying molecular alterations [33]. Clinical presentations show HCC is seropositive with AFP, or AFP-L3 or DCP with only some degrees of overlapping patterns although some HCCs are seropositive with all the current available HCC biomarkers [29, 30]. How the phenotypic variation patterns related to biologic behavior can be interpreted for directing treatments remains to be determined. This also suggests that some serum biomarkers complementary to the existing ones remain to be discovered. But it should be clear that usefulness of any single biomarker in HCC surveillance is limited.

Combined use of serum biomarkers can maximize the clinical sensitivity (or specificity depending decision rule). Overall test results can be registered as positive using algorithm of "OR" or "AND" rule depending on the clinical context [34]. This offers a rationale and testable hypothesis for using multiple serum biomarkers simultaneously in hoping for achieving higher clinical sensitivity and/or specificity. As a matter of fact, recent studies did have provided "prove of concept" of such approach [35, 36].

12.1.3 Assay Calibration: From Data to Information

Information must be extracted from the data in order for the data become useful or actionable to clinicians. In this sense, the information is the data which are interpretable for further clinical actions [5]. Structured data from quantitative measurements have intrinsic values such as measurement concentrations of cancer biomarkers CA125 or CA19-9. These clinical data may not have any information simply because we do not know what they are actually meant. For serum biomarkers, one approach for extracting meaningful information from measurement data are through comparison to a Gold Standard which could be histology from biopsied or surgical specimens, or in the case of early diagnosis of HCC, clinical diagnosis based on dynamic imaging modalities such as four phase contrast CT or MRI. Tissue morphology by staining have provided disease diagnostic standard for human diseases. Medical sciences have evolved in recent years for HCC diagnosis. Clinical diagnosis and decision for early intervention can be made based on clinical diagnosis which relied on dynamic CT or MRI. It is worth noting that tumor size has been integrated in the HCC definition in the AASLD clinical practice guideline for HCC management.

Until now, the cancer biomarker assays have not been calibrated based on tumor size as a clinical parameter as a gold standard. This has led to spectrum bias in many studies reported of performance characteristics using the serum biomarkers. The possibility of the use of cancer biomarkers calibrated based on smaller tumor size from imaging would represent a paradigm

shift in medical diagnosis which can set the diagnostic threshold lower for detecting earlier stage of HCC. How effective these serum biomarkers are for early detection remains to be determined by clinical studies in relevant clinical contexts.

12.1.4 Level of Evidences: From Clinical Validity to Utility

Safety and effectiveness are the basis of the FDA clearance and approval of the serum biomarkers for marketing in the U.S. which constitute the regulatory framework for medical devices. The evidences can be obtained from observational study or clinical experiment. For regulatory clearance or approval, observational study with clear intended use and indication for use in retrospective or prospective designs can be used to collect the validation data for demonstrating the safety and effectiveness. The most commonly used clinical parameters are sensitivity and specificity for effectiveness and false positive and false negative for safety which should be evaluated in light of a specific clinical context i.e. the intended use and indication for use as proposed for the serum biomarkers.

Pepes et al. have proposed five phases of biomarker development for early detection of cancer: (a) preclinical exploratory studies (phase 1); (b) clinical assay and validation (phase 2); (c) retrospective longitudinal (phase 3); (d) prospective screening (phase 4); and (e) cancer control (phase 5) [37]. These ordered phases of biomarker development have provided a framework for rational development and clinical adoption of serum biomarkers for cancer early detection.

The data from different phases of the evaluation stage present different levels of evidences. National Comprehensive Cancer Network (NCCN) Task Force on Evaluating Clinical Utility of Tumor Markers in Oncology has affirmed the level of evidence in their newly released practice guideline on cancer serum biomarker evaluation [38]. A system of the levels of evidence has been outlined as in Table 12.1.

Most clinical studies leading to FDA clearance or approval likely remain in phase 3 developmental stage providing relatively low levels of evidence for clinical utility. It would be challenging in convincing clinicians that the tumor markers with regulatory clearance or approval have clinical utilities satisfying their unmet medical needs. Clinically, the only reason for diagnostic testing is treatment decision [39]. A biomarker would have clinical utilities if it can direct treatment based on high level of evidences from well-designed clinical studies. However, clinical utility can be suggested by observational study with robust designs [40].

Table 12.1 Tumor marker utility grading system level of evidence

Level	Interpretation
I	Prospective, marker primary objective, well-powered or meta-analysis
II	Prospective, marker the secondary objective
III	Retrospective, outcomes, multivariate analysis (most currently published marker studies are level of evidence III)
IV	Retrospective, outcomes, univariate analysis
V	Retrospective, correlation with other marker, no outcomes

12.2 HCC: Risk Factors and Clinical Measurements

12.2.1 Chronic Hepatitis, Liver Fibrosis and Cirrhosis

Chronic liver diseases in forms of liver fibrosis or cirrhosis are precursor of liver failure and HCC, the end stages of the liver disease. Worldwide the most common causes of chronic liver disease are chronic hepatitis B and C virus infection. After decades of the initial HBV or HCV infection, a pathological process in liver characterized by stepwise progressions of chronic liver disease could lead to fibrosis and cirrhosis, eventually to liver failure or primary liver cancer. The hepatitis B virus B and C virus infection are the major risk factors for HCC. Relative risk (RR) for HCC with HBV infection is approximately 100 compared to

non-carriers; in cirrhotic HBV carrier, the RR was 961 compared to uninfected controls [8]. HCC risk was also drastically increased to 20–200 times in HCV-infected patients compared to HCV-negative controls [41]. The conversion rate of HCC is 1–6 % per year among the chronic hepatitis patients with cirrhosis [6]. It has been reported that obesity and diabetes from metabolic syndrome are associated with HCC as the emerging risk factor of HCC [42, 43].

There are multiple clinical staging systems for liver cancer for predicting the prognosis of HCC. The major ones include American Joint Commission on Cancer (AJCC) Tumor-Node-Metastasis (TNM) system, the Barcelona Clinic Liver Cancer (BCLC) System, Cancer of the Liver Italian Program (CLIP), and the Okuda System, etc. [14]. Although none of these scoring systems have been universally accepted, they invariably incorporate some most important considerations for survival of HCC, namely (a) severity of the underlying liver disease; (b) tumor size; (c) intrahepatic micro-invasion; and (d) metastasis [44]. In a retrospective cohort study published in 2009, Nathan et al. compared six major staging systems for HCC with an early HCC prognostic score [45], and concluded that an early HCC prognostic score is superior to the AJCC TNM system for predicting survival of patients with early HCC after liver resection or liver transplantation. The investigators found that all the major HCC staging systems performed poorly in patients with early HCC. This is likely due to the fact that liver functions are not accounted for in the staging schemes [14].

AASLD Clinical Practice Guideline on HCC Management recommends use of US every 3–6 months for HCC surveillance for patients at risk for HCC. NCCN Clinical Practice Guideline in Oncology on Hepatocellular Carcinoma recommends utilizing both US and serum biomarker AFP for screening patients in an interval of every 6 months. Overseas the J-HCC/Japan Society of Hepatology (JSH) recommends use of serum biomarkers AFP, AFP-L3 and DCP every 3–4 months for the patients at high risk for HCC in addition to US [20].

Other imaging modalities such as dynamic contrast CT and MRI have been used for annual surveillance of patients at risk for HCC although they tend to be utilized more in confirmative diagnosis, especially in tertiary health care settings. The imaging modalities are technically demanding and not as convenient as testing of patient specimens being drawn and sent to reference laboratories. Similar to serum biomarkers, the imaging modalities are, in general, of relatively low sensitivity but of high specificity. In recent years, the treatments of HCC can be initiated according to the clinical diagnosis provided by the vascular characteristics on imaging of the liver malignancies, thereby representing a new framework of clinical utility of biomarkers.

12.2.2 Clinical Measurements: Enzyme Aberration in Glycosylation and Carboxylation

Clinical measurements of serum biomarkers have focused on changes of protein concentration in circulation. However, variations in protein concentration such as hormones and growth factors are thought to be mostly physiological phenomenon reflecting feedback regulations instead of pathological presentation [5]. The operating ranges of most of the cancer biomarker assays are probably well above the concentration gradients of many biologically important molecules in cancer early development [46]. Furthermore, although in biology, information flows from DNA to RNA, to proteins, it is post-translational modifications such as protein phosphorylation and glycosylation that empowers protein molecules with functional significance.

At the molecular levels, AFP-L3 is a glycosylation variant of AFP with α-1,6 core fucosylation on reducing terminus of N-acetylglucosamine of AFP molecule which is the AFP fraction reactive to lectin Lens culinaris agglutinin [2]. The elevation of AFP-L3 in HCC results from overexpression of fucosyltransferase Fut 8 which is responsible for core-fucosylation of proteins in the liver and other enzymes facilitating synthesis of GDP-glucose, the substrate of the fucosyltransferase [47]. Fucosylation is one of the most common post-translational modifications of

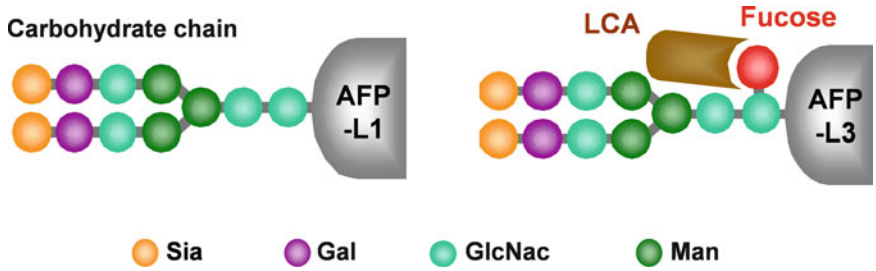

Fig. 12.1 Alpha-fetoprotein (AFP) isoforms: AFP-L1 (*left*), and AFP-L3 (*right*). Note: Sialic acid (*Sia*); Galatose (*Gal*); N-Acetylglucosamine (*GlcNac*); Mannose (*Man*) (http://www.wakodiagnostics.com/afpl3test.html)

proteins in physiology. Increase in fucosylation has also been reported in inflammation and cancer. Fucosylated glycoproteins are involved in biological functions of adhesion molecules and growth factor receptors through Notch signaling [48]. Core fucosylation is reported crucial for cytokine receptor activation [49]. The increased concentration of AFP-L3 is also due to increased release of the AFP-L3 from hepatocytes in HCC into plasma which is normally secreted into the bile duct (Fig. 12.1) [50].

Patients with primary malignant hepatic tumors seropositive for AFP-L3 and low AFP concentrations appear of unique clinicopathologic features. It is reported these cancers have a higher incidence of non-HCC primary liver cancer derived from cholangiocytes. They also had a high frequency of poorly differentiated tumors and sarcomatous changes, and showed a poor prognosis [51]. HCC patients who were positive for AFP-L3 and negative for DCP demonstrated histopathologic features of more advanced HCC compared with those who were seropositive for DCP alone such as infiltrative growth with an irregular margin and showing poorly differentiation of the HCC [52]. In fact, Okuda et al. found that a subgroup of intrahepatic cholangiocarcinoma (ICC) are seropositive for AFP-L3 and those with combined hepatocellular and cholangiocarcinoma have features close to HCC. The investigators thought that these liver cancers may be different from the ICC which is seropositive with CA19-9 [53]. This suggests that AFP-L3 seropositive HCC is a subtype of primary liver cancer with more aggressive behaviors.

DCP or proteins induced by vitamin K absence or antagonist-II (PIVKA II) is an abnormal form

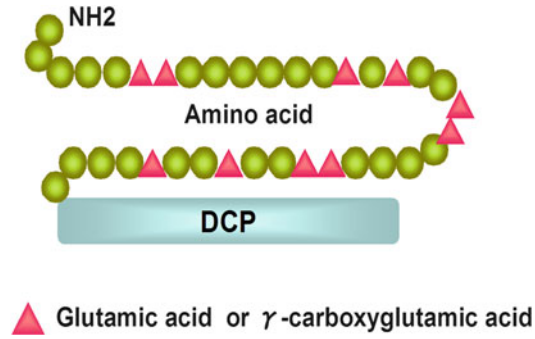

Fig. 12.2 Des-gamma-carboxy prothrombin (DCP) (http://www.wakodiagnostics.com/pivka_dcptest.html)

of the coagulation protein produced by the liver in HCC. Prothrombin is also known as the Coagulation Factor II of the blood coagulation cascade. In normal liver, the prothrombin undergoes post-translational carboxylation before release into the peripheral blood. The carboxylation converts specific amino-terminal glutamic acid residues to γ-carboxyglutamic acid [54]. The vitamin K dependent carboxylase responsible for the carboxylation is absent in malignant cells, and an abnormal prothrombin with all or some of unconverted glutamic acid is released into the circulation instead. The non-carboxylated form i.e. DCP is a biomarker for HCC (Fig. 12.2). Some subgroups of HCC, probably due to malfunction of carboxylase, secrete the unmodified precursor, DCP. In a study comparing hypervascular and hypovacular HCC, Matsubara et al. found that DCP production is associated with tumor angiogenesis of HCC [55]. Yuan et al. reported that the DCP levels in HCC tissue with portal vein invasion were significantly greater than in HCC tissues without portal vein invasion

[56]. In addition, recent studies have revealed that DCP functions as a growth factor and might play significant roles in cancer progression [57]. Durazo et al. showed that DCP has a direct correlation to tumor size in patients with single lesion [58].

It has been suggested that the both AFP-L3 and DCP are associated with tumor aggressiveness of HCC [59]. In particular, AFP-L3 is related to progression from moderately differentiated to poorly differentiated HCC, whereas DCP is more specific to vascular invasion and is therefore likely to be a useful indicator of vascular invasion [60].

12.2.3 History of AFP-L3 Developments and Technical Features

AFP-L3 is a glycoform with core fucose glycosylation. Based on its affinity to lectin Len culinaris agglutinin, AFP can be sub-fractionated into three distinctive species i.e. L1, L2 and L3 according to their reactivity to Lens culinaris of migration pattern of affinity electrophoresis. Investigations found that the L1 was elevated in inflammation of liver. The L3 is tumor-specific for HCC. The micro-heterogeneity of the glycan structural variation between AFP-L1 and AFP-L3 is due to presence of an α-1,6, core fucose at the reducing end of the N-acetylglucosamine of AFP [2].

The first clinical laboratory assay on AFP-L3 was developed with a lectin-affinity electrophoresis method. The lectin lens culinaris was used to separate the three fractions of the AFP based on its reactivity to the agglutinin. Detection of the L3 ratio in percentage to L1 was achieved by dye-labeled antibodies and quantification by densitometry. An automated assay was developed for clinical use in 1997 in Japan on a liquid phase binding immunoassay platform, (LiBASys). The AFP-L3% reading was generated when AFP is >10 ng/mL with a minimal detectable limit of AFP-L3 at 0.8 ng/mL [61]. The LiBASys AFP/AFP-L3 assays were cleared by the FDA for risk assessment of HCC in the U.S. in April of 2005. Subsequently, the DCP assay was also cleared by the regulatory agency for the same indication for use, and was added to the test menu. Since then the assay technologies have continued to evolve. Since 2009, the assays have migrated to a state-of-the-art immunoassay platform based on microfluidic chip as an electrokinectic analyte transport assay (EATA). With deployment of the second generation of the assay instrument, the analytical sensitivity has increased with further diminishing the minimal detection limit to 0.3 ng/mL of AFP-L3. The assay range of AFPL3% has been extended from 0.6 to 1000 ng/mL of AFP [62]. The assay system can provide accurate and precise percent ratio AFP-L3 reading over the entire assay range of AFP from 0.6 to 1000 ng/mL. This has rendered significant improvement in assay sensitivity while maintaining the clinical specificity facilitating clinical applications for early detection of smaller HCC. Furthermore, the fully automated features in designs of the analyzer have greatly shortened the assay turnaround time to less than 10 min [62].

12.2.4 Technical Features of Chip-based Microfluidic Assay and Analytical Performance

The EATA immunoassay on the microfluidic chip immunoassay platform can carry out reagent and sample mixing, concentration, reaction, and also can integrate all other assay steps on chip. The microfluidic chip was made with precision injection modeled from poly(methyl methacrylate-PMMA) plastic resin, and the channels were formed by bonding of plastic film to the modeled chip. PMMA has no ionizable group. Using a non-charged substrate has minimized electrostatic interactions between the analytes and the micro-channel's surface; and helped to reduce the electroosmotic flow (EOF) which can assists in clean and clinical assay grade quality separation of the immunocomplexes in the capillary electrophoresis on chip [63].

The EATA immunoassay is highly sensitive using <10 nL of actual serum size of specimen per measurement. The underpinning technology of high analytical therefore the high clinical

sensitivity is isotachophoresis (ITP) which allows target analyte to be highly concentrated prior to detection using laser-activated immunophorescence dyes. ITP has been demonstrated to enhance analyte concentration by as much as three orders of magnitude enhancing the analytical sensitivity of the assays [63].

DNA-conjugated antibody has been employed for precisely control, adjustment and fine-tuning the electrophorectic mobility of immunocomplexes by varying the length of the conjugated oligonucleotides. The immunocomplex is also bound to another fluorescent-labeled antibody specific for the analyte which is under controlled for unidirectional migration together with the specimen and reagents from beginning to end of the assaying process on chip [63].

The advanced technical features of the microfluidic assay platform are attributed to the exceptional performance characteristics in analytical validations. The reproducibility of the assays is demonstrated that the coefficient variation (CV) is within 2 % for AFP and 3 % for AFP-L3. The assays' imprecision is reduced to minimum. The proportional bias has been shown within 2–3 % in comparison to the electrophoresis and LiBASys methods. The systemic bias of the assays in general is less than 5–6 %. In serial dilution experiments, the AFP-L3% has been shown held in a constant level over the entire assay range with changes in AFP concentrations in a reportable range from 0.6 to 1000 ng/mL [62].

12.3 Clinical Performance: Parameters and Interpretations

12.3.1 Clinical Validity: Parameters

The clinical parameters most commonly used in evaluating and demonstrating the clinical validity are sensitivity and specificity which are relatively unaffected by prevalence of the disease in population. Since the clinical sensitivity and specificity of a test are trade-offs depending on the assay cut-off value which should be determined and chosen according to the indication for use of the assay. It is unreasonable to expect an assay to have both very high sensitivity and specificity since human disease is a spectrum in development, especially the degenerative disease such as cardiovascular, metabolic, and malignant disorders. The purpose of diagnosis is to treat patients. The threshold of making definitive diagnosis is a balance between costs and benefits [64]. The benefit is therefore also depending on effective treatments available. Of importance in assessing the clinical validity and utility of the assays is the indication for use and the clinical context the assay is applied. For example, diagnosis tools such as serum biomarkers could be used for rule-in or a rule-out diagnosis. The selection of a clinically valid cut-off for assay requires a clinical context. For the rule-in diagnosis, a positive testing would be more valuable with high specificity to avoid unacceptable level of false positive results, whereas for rule-out diagnosis, negative testing result is more important with high sensitivity and low false negative rate.

In general, the microfluidic chip-based AFP-L3 and DCP assays are highly specific for early HCC although the clinical sensitivity and specificity vary by the cut-off threshold chosen and by tumor size which the assays designed to detect. These HCC usually featured by low AFP concentration <20 ng/mL. For assay with high clinical sensitivity, negative test results are more informative. The assay with high NPV from high sensitivity is used for screening or surveillance in clinics. The seronegative result can rule out suspicious HCC. In contrast, seropositive data of AFP-L3 and DCP should be cautious in interpretation since the potential false positive results need to be teased out. The performance of the AFP, AFP-L3 and DCP assay on microfluidic assay platform of *μTASWako i30* are summarized in Table 12.2 [65–67].

The clinical performance characteristics as shown in the Table are clearly influenced by the cut-off selected according to the proposed indication for use of the medical devices. They could be affected as well by tumor characteristics such as tumor size which the medical devices are expected to detect, and the staging system used for categorizing the malignant disease. Since

Table 12.2 Clinical performance of AFP-L3, and DCP assays on *μTASWako i30*[a]

HCC serum marker	Sensitivity	Specificity
AFP-L3 (%)		
>1 %	68 %	81 %
>5 %	40–53 %	54–87 %
>7 %	24–41 %	92 %
>10 %	12–21 %	97–98 %
>15 %	9 %	97 %
AFP-L3 (5 %)		
≤2 cm	37 %	
>2 and ≤3 cm	46 %	
>2 and ≤3 cm	44 %	
>5 cm	47 %	
DCP (mAU/mL)		
>40	56 %	95 %
DCP (40 mAU/mL)		
≤2 cm	24 %	
>2 and ≤3 cm	52 %	
>2 and ≤3 cm	64 %	
>5 cm	78 %	

[a]All study subjects had AFP < 20 ng/mL

dynamic imaging has been widely accepted for clinical diagnosis of HCC, the disease definition of HCC should be specified including information of imaging modality and the contrast reagents used. Also because of the background liver cirrhosis, size of the tumor nodule for definitive HCC diagnosis is also important for the performance characterization.

The serum biomarkers, when used individually especially at the lower cutoff, have demonstrated comparable performance to ultrasonography. Recent research data have indicated that serum biomarkers when used in parallel or simultaneously, can maximize the clinical sensitivity while maintaining clinically acceptable specificity to meet the operating requirement of performance for HCC surveillance [36, 66]. Feng reported that combined AFP and DCP with cut-off threshold set at >6 ng/mL and >100 mAU/mL, respectively, can significantly improve the clinical sensitivity of the overall testing to 94.5 % [34]. Separately, Volk et al. also demonstrated the similar results for early stage HCC [35].

Recent study data further indicated that the combined use of all three current available HCC serum biomarkers can improve performance of the overall test result for early HCC. Hanaoka [36] showed that the overall sensitivity of AFPL3 plus DCP can be boosted to 78 % while maintaining the specificity basically the same as the respective serum biomarker at 86 %. For early HCC i.e. those with tumor nodule <2 cm in diameter, the sensitivity of the biomarkers were 24 % for DCP (using 40 mAU/mL as a cut-off) and 37 % for AFP-L3 (using 5 % as a cut-off) (Table 12.2), respectively. The relatively low sensitivities are not unexpected which may be due to tumor biology of the early HCC since the HCC is highly heterogeneous [68] as being reflective of discernible and yet non-overlapping expression patterns of the HCC biomarkers. This is echoed by Sherman M. who reported that 20–80 % of the HCC did not produce AFP depending on the tumor size at diagnosis [31]. Of note is that breast cancer has multiple subgroups with distinct clinical outcomes which may also be represented in HCC as well [14]. This further implies that the expectation of any single laboratory testing can achieve extremely high sensitivity while maintaining high clinical specificity may be unrealistic. Therefore although high clinical sensitivity is desirable for cancer screening and surveillance, it is a complex issue involving not only assay performance but also related to the gold standard employed for the performance comparison, as well as to intrinsic tumor biology.

Many serum HCC biomarkers of potential clinical usefulness have been found such as Glypican-3, Golgi protein 73 (GP73), and osteopontin [69, 70]. Due largely to the intrinsic biological heterogeneity, some HCC were not detected by every serum HCC biomarker. Thereby the performance characteristics especially the sensitivity would vary significantly among different serum biomarkers. It is expected that this performance gap would be narrowed with additional new discoveries of HCC serum biomarkers followed by parallel applications of multiple serum biomarkers in the testing algorithm. Recently, Shen et al. reported that a new serum biomarker of Dickkopf-1 (DKK1) could complement AFP in detecting HCC subtypes in patients of sero-negative AFP [71].

AFP-L3 and DCP are highly specific cancer biomarkers. AFP is a tissue specific embryonic antigen. It is re-expressed in some human cancers distinctively such as in testicular and primary liver cancer. It is clear that the elevation of AFP is more related to tissue necroinflamamtory reaction of hepatocytes of the underlying chronic viral infections [3]. Empirical data demonstrated AFP is consists of different glycoforms with reactivity to Lens culinaris. AFP-L1 is the major AFP fraction presenting in the necroinflammatory reaction that would likely elevated at liver tissue regeneration after necrosis. AFP-L3 is cancer-specific [3]. The practical implications for the finding of AFP-L3 is HCC-specific would be far-reaching in risk assessment, screening or surveillance, and diagnosis. Other potential clinical utilities of the serum biomarkers could include predicting prognosis and monitoring recurrence after treatments in surgical resection, radiofrequency ablation (RFA), and liver transplantation. It has been reported that the AFP-L3 concentration has fallen significantly beyond half-life of the serum protein in circulation in patients treated successfully by surgery and RFA in those patients presumably had not intrahepatic invasion or metastasis [72, 73]. Retrospective data analysis also suggested the patient cohort with low AFP-L3 < 5 % who had undergone successful surgical resection showed better long term survival compared to those with AFP-L3 > 5 % [68]. In comparison, AFP and DCP have been utilized as prognostic biomarkers in liver transplant for predicting recurrence and outcomes in the same study, but they were not associated with favorable outcome in survival [66]. Therefore, it appears AFP-L3 could be used for directing treatment and predicting prognosis of the treatments.

12.3.2 Test Interpretation: The Caveats

When interpreting test results of AFP-L3 and DCP, keep in mind that the sensitivity and specificity are conditional probabilities. The clinical parameters of sensitivity and specificity are useful but limited for at least by two reasons. The first is these are population level statistics. They cannot be easily applied in individual patient because one has to assume that the clinical truth about the disease status is already known. This is not true in clinical decision making using the biomarkers [74]. In addition, verification bias could affect the performance characteristics of a diagnostic device if gold standard is not applied across the entire study population for assessing the assay performance characterizations. This could happen when the test negative patients in a study have no imaging data to confirm the lack of HCC. This is not unusual in many oncology device investigations. For instance, due to ethical consideration, some patients in a study with negative lab testing results may not be subject to the same rigorous verification of disease status by tissue biopsy as the positive cases were. This is not trivial in clinical validation of oncology study.

In contrast, positive and negative predictive value (PPV and NPV) of testing could offer useful information for the assessment of risk or probability of HCC at individual level. However, PPV and NPV could be affected by pre-test probability i.e. the prevalence of the disease. For HCC, disease prevalence is relatively low in population, approximately 5 % among the patients at risk for HCC at least in North America. Under such circumstance, a diagnostic test may be unproductive in terms of the information yield from the testing procedure.

This brings us to another aspect of the testing utilization of the AFP-L3 and DCP in surveillance of HCC risk. Surveillance is repeat use of screening for patients at risk for HCC which is a targeted screening using AFP-L3 and DCP assay in patients of chronic hepatitis and cirrhosis [15]. Outside the United States, surveillance is established in medical protocol for HCC management in some countries. For example, Japanese government has sponsored and endorsed the practice guideline of HCC surveillance employing US and the novel HCC serum biomarkers [20]. Periodic and serial sampling is imperative for accurate assessment of the HCC risk in clinical decision making using the serum biomarkers in surveillance. In this case, changes of the serum

biomarkers in value compared to baseline may make more clinical senses than simply look at an individual test result at any random time point. The assay interpretation in surveillance should rely on trending of the measurement values of the serum biomarker variation overtime. Multiple readings of AFP-L3 and DCP may mitigate the risk of false negative testing result or can even help to address the concern of false positive result of the tests which may be a more efficient way to identify the patients of early HCC.

In evaluating of performance of a diagnostic test, a test is informative if sensitivity plus specificity is >1.0 or if PPV is greater than prevalence [75]. But its acceptance in medical practice in HCC management will depend on understanding of what is the actionable information derived from the testing procedure to answer the question of whether a patient should be treated. For most diagnostic testing, such information could be only feasible from post-market or phase 4 clinical study design, or from user experiences since the HCC nodules have to be found in order to be treated.

HCC is a future event in the context of risk assessment. The clinical parameters appropriate for this purpose are relative risk (RR) and odds ratio (OR). The value of RR and OR > 1.0 with 95 % confidence interval not bracketing 1.0 is considered statistically significant. For risk assessment, it is meaningful if these values are much greater than 1.0 [76]. Furthermore, a timeframe associated with the risk implied should also be specified for RR. The RR of HCC for positive AFP-L3 and DCP is 10.6 and 4.8, respectively when the cut-off of AFP-L3 is set at10 % and DCP at 7.5 ng/mL (product package inserts, Wako Life Sciences, Inc., Mountain View, CA) indicating that the risk of HCC of seropositive AFP-L3 and DCP is 10 and 5 times higher, respectively, in next 2 years compared to those with the assay results remain negative.

12.4 Summary: Potential Clinical Utilities

AFP-L3 and DCP are the serum biomarkers with FDA clearance for marketing in the United States for the indication for use of risk assessment of HCC development in conjunction with other clinical information. The criteria of the regulatory clearance are safety and effectiveness of the device for the stated indication for use. The safety of the device for use in risk assessment is further ensured by the statement that the devices should be used in conjunction with other clinical diagnostic modalities for decision making. Fundamentally, the patient safety is driven by the biological nature of the serum biomarkers i.e. their disease-specificities in general and tissue specificities in particular. High specificity implies that elevation of AFP-L3 and DCP in circulation is pathognomonic for HCC irrespective of the gold standard in use for performance comparison. High specificity is also indicative of the devices are of low false positive rate. A positive assay alerts of early HCC development in the patients with excessive risks for HCC because the high PPV of such testing is revealing, and the patients should be followed-up closely for confirmation. The dilemma facing clinicians in interpreting the assay results is that they will have to find the tumor nodule in order to initiate medical or surgical interventions in a timely manner. Technology advances in clinical measurements sometime indeed pose unintended challenges instead of immediate answers to clinicians.

While the high specificity of the HCC biomarkers could be indicative of high risk of HCC, the high clinical sensitivity is desirable but it should be secondary to clinical specificity. The improvement in clinical sensitivity was largely limited by the assay technology in the past, but also by the gold standard used for performance comparison now. With advancement in technologies for clinical measurements, as demonstrated in the cases of AFP-L3 and DCP, it has become apparent that value of the clinical laboratory tests will depend on understanding of the clinical significance of the testing results with clear clinical utilities. User experience should be important in delineating the clinical usefulness of the HCC biomarkers. It should also be pointed out that the clinical sensitivity and specificity can be improved significantly by serial sampling and the combined use of the cancer biomarkers in parallel or in tandem in algorithms or by adding newer or more sensitive biomarkers in the future.

The complexities of human biology in disease developments unraveled by the improvements in measurement technologies suggest further collaborative efforts for determining the potential clinical utilities of the novel cancer biomarkers are necessary. Perhaps biomedical informatics can come to our helps in the near future in this regard with integrative data modeling tools for clinical algorithms in clinical decision making.

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