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DNA markers in molecular diagnostics for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the one of the leading causes of cancer mortality in the world, mainly due to the difficulty of early detection and limited therapeutic options. The implementation of HCC surveillance programs in well-defined, high-risk populations were only able to detect about 40–50% of HCC at curative stages (Barcelona Clinic Liver Cancer stages 0 & 1) due to the low sensitivities of the current screening methods. The advance of sequencing technologies has identified numerous modifications as potential candidate DNA markers for diagnosis/surveillance. Here we aim to provide an overview of the DNA alterations that result in activation of cancer pathways known to potentially drive HCC carcinogenesis and to summarize performance characteristics of each DNA marker in the periphery (blood or urine) for HCC screening.

Keywords

circulating DNA biomarkers; HBV-associated HCC DNA markers; HCC DNA markers; HCC surveillance and early detection; hepatocellular carcinoma; non-invasive

Hepatocellular carcinoma (HCC) represents 90% of all adult primary liver cancers and is one of the leading causes of cancer mortality in the world, responsible for over 746,000 deaths in 2012 and accounting for 9.1% of the global cancer mortality rate [1]. While overall cancer mortality has decreased, liver cancer related mortality has been rising steadily by 2.4% yearly, with 782,000 new HCC cases each year [1]. This high mortality rate is mainly due to limited therapeutic options and difficulty in the early detection, thus undermining the prognosis [2].

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The 5-year survival rate for HCC is 29% when detected at an early localized stage, 10% at a regional stage and as low as 3% at a distant metastasized stage [3,4]. Up to 90% of HCC cases arise in individuals with an established risk factor. Given that there is a long period, sometimes over a decade in length, between the establishment of risk factors and the development of HCC, surveillance of patients at risk for HCC is a critical component of any measure adopted for early detection of cancer and improvement of patient prognosis, as recommended by both the American Association for Study of Liver Diseases (AASLD) and the European Association for Study of Liver Diseases (EASL) [2,5,6]. Despite the implementation of HCC surveillance programs in the well-defined, high-risk populations, early detection of HCC remains difficult to achieve with current screening methods, due to low sensitivities. The most widely used biomarker for HCC screening is the serum level of α -fetoprotein (AFP), which has a sensitivity of 40–60% for all stages and 40–50% for early stages HCC [2,7,8]. Thus, the need is urgent for biomarkers with increased sensitivities for the early detection of HCC.

Like other cancers, HCC is a disease of the genome, caused by genetic and epigenetic DNA modifications [9–11]. Identification of the DNA modifications (mutation or methylation) underlying the development of HCC should permit unambiguous diagnosis of HCC cases. The introduction of next generation sequencing (NGS) has led to the identification of many of such modifications, associating with HCC tissues. A significant amount of work has focused on distinguishing the drivers from the passengers of these DNA modifications, in combination with studying the pathways involved in carcinogenesis to determine how these DNA modifications can be used as potential markers for HCC detection [11–19]. However, these candidate DNA markers will not be useful for either diagnosis or surveillance unless they can be detected in the peripherals, without liver biopsy. The objectives of this article are to provide an overview of DNA alterations that result in activation of cancer pathways known to potentially drive HCC carcinogenesis and to summarize performance characteristics of each DNA marker as a potential marker for HCC screening. Special attention is drawn to the markers that have been previously detected in the peripherals (blood or urine) in association with HCC. Finally, we discuss how these HCC markers can be used in HCC surveillance and personalized management of patients.

HCC surveillance

Almost 90% of HCC cases arise in individuals with an established risk factor. These risk factors include hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol intake, aflatoxin B1 exposure, hemochromatosis, α -1-antitrypsin deficiency, primary biliary cirrhosis and non-alcoholic fatty liver disease [2,5,20]. An HCC-surveillance program has been recommended by both AASLD and EASL to patients with high-risk factors, in order to detect HCC earlier for better prognosis [2,5]. The current recommended test for surveillance of HCC is an abdominal ultrasound performed by an experienced operator every 6 months, in subjects at risk for HCC. Ultrasound, as a screening test, has a sensitivity in the range of 40–89% and a specificity of 90%. This wide range of sensitivity is mainly due to the skill of the operator and the challenge of detecting HCC in a background of cirrhotic liver with micro- and macro-regenerative nodules [5,21–23]. Serum AFP levels is one of the most commonly performed and best-studied serological test. At a cutoff of 20 ng/ml, serum AFP has a

sensitivity of 40–60% and a specificity of 90%, but an unacceptable positive predictive value of 25% at 5% disease prevalence [7]. Thus, AFP as a screening test at a 20 ng/ml cutoff has both a high false positive rate and a poor efficacy for surveillance, and is therefore not recommended as a surveillance tool, either alone or in combination with ultrasound, by both AASLD and EASL. Other serological tests such as des- γ -carboxy prothrombin and AFP-L3 fraction have low sensitivities and are also not recommended for screening [5,24,25]. Additional tests include CT scan and MRI, which are both sensitive imaging methods, but they too are not used for HCC-surveillance because of the high cost and the inherent risk of radiation. With the current surveillance program for HCC detection, an average of 50% of cases are identified at early stages of HCC [26]. Consequently, better screening tests are needed to improve the efficacy of HCC surveillance.

Potentials of circulating DNA markers for HCC surveillance & the early detection

Malignant transformation of hepatocytes occurs when DNA alterations either activate or inactivate certain cancer pathways that induce uncontrolled growth of the cells, or clonal expansion, which is initiated from primary alterations that occur in genes as ‘drivers’. The development of HCC, as with other solid tumors, is believed to require the dysregulation of at least three core cellular pathways (cell cycle, apoptosis/cell survival, genome maintenance) within the cell [27–30]. Thus, identification of the DNA modifications underlying the development of HCC should permit unambiguous diagnosis of HCC. A great deal of work has been devoted to identifying such DNA modifications as potential biomarkers for the early detection of cancer. In general, the number of candidate cancer biomarkers has exploded since the introduction of genome wide sequencing of diseased tissue DNA, using NGS [11–15,18,19,31–33] and genome wide methylation study using methylation arrays [34–37]. However, such identification will only be useful for cancer screening and the early detection of cancer, if it can be done in the periphery in a non-invasive or minimally invasive manner.

DNA containing cancer ‘signatures’ (mutations or hypermethylation) has been found in the plasma, serum and urine of patients with many cancers, including HCC. In almost all instances where tumor tissue was available, the tumor associated DNA modifications in the plasma [38–40] or urine [41–43] were generally consistent to those modifications detected in the primary tumor, demonstrating that the tumor-derived DNA can be detected in the circulation via blood or urine, if a tumor is present. Several reviews have addressed the possible mechanisms of the presence of cell-free tumor DNA in the circulation [44–47]. Briefly, this cell-free circulating tumor DNA could be either from primary tumor or from circulating tumor cells due to cell death by either necrosis or apoptosis, or even microvesicle-released DNA in the circulation originating from tumor cells. Regardless of the various sources of cell-free circulating tumor DNA, the presence of tumor DNA in circulation provides a great promise to use blood or urine as a liquid biopsy to profile cancer genetics for cancer screening or early detection [44,48–52].

One advantage of using DNA biomarkers is that an HCC DNA test can be very sensitive if the test can detect all the possible DNA modifications that drive hepatocarcinogenesis in a

non invasive or minimally invasive manner. Furthermore, DNA tests can also provide information on cancer genetics, helping to determine a possible prognosis and even tailor personalized treatments for patients, if a tumor is detected. Another clear advantage of using circulating DNA biomarkers for HCC screening as the detection of the DNA markers are not affected by the background liver disease, location of the lesions, number of nodules or obesity, which are all technical challenges for ultrasound. Thus, if using DNA markers alone does not provide sufficient sensitivity for HCC screening, then these markers can at least increase the performance of ultrasound .

The major challenge, however, is the specificity of circulating DNA markers for HCC. As mentioned, the sensitivity of the test can be extremely high, thus the specificity of the test will always be a challenge because most driver modifications are not liver-cancer specific. For instance, mutations of *TP53* or methylations of tumor suppressors (such as the *adenomatous polyposis coli* gene [*APC*], *RASSF1A* and *CDKN2A*) are found not only in HCC, but also in many other cancers [53-58]. Fortunately, the specificity of the test can be improved if organ-specific markers, such as *TP53 249T* mutations or HBV-associated HCC DNA markers for patients with HBV-infections (discussed in a latter section), are included [42,59–61]. Taking advantage of a high sensitivity for early detection in a minimally or non invasive manner, the circulating-DNA markers, remain extremely valuable as a screening tool, even with compromised specificity. These markers can be used to guide patients toward more sophisticated imaging diagnosis, or be used in combination with patient history, to provide a molecular characterization of the tumor for better management, if the tumor is diagnosed.

Strategy for constructing a panel of multiple DNA makers for HCC screening

As discussed, DNA markers should provide high sensitivity for HCC screening, if a test can detect all the drivers of hepatocarcinogenesis. HCC has a multifactorial etiology resulting in a disease with high heterogeneity, as different risk factors disrupt different signaling pathways. Thus, a single DNA marker is unlikely to have sufficient sensitivity for HCC screening, whereas a panel of biomarkers encompassing the frequently altered genes in the key pathways associated with HCC patho genesis would be needed to provide sufficient sensitivity to detect most of the HCC. Previous studies have shown that mutations in cancer pathways exhibit an ‘exclusivity principle’, where if a certain gene is mutated in a pathway, other genes in that pathway are excluded from mutations [62–64]. The best known example is the exclusive existence of the *K-ras* and *BRAF* mutations in a tumor since these two genes are within the Ras pathway [65]. Similarly, in HCC, studies have suggested that the *CTNNB1*, *AXIN1* and *APC* gene mutations are mutually exclusive [18,66,67]. Although correlated biomarkers (biomarkers that belong to only one pathway) can underscore the importance of a biological pathway, they often do not provide a substantial increase in predictive value when they are combined in a panel, unless the pathway is an essential pathway [68–70]. Thus, a large number of correlated biomarkers are substantially less informative than a small number of uncorrelated biomarkers. For instance, when we combined four uncorrelated biomarkers, the methylated *APC* gene (*mAPC*), the methylated

RASSF1A gene (*mRASSF1A*), the methylated glutathione S-transferase P1 gene (*mGSTP1*) and *TP53* 249T mutation in one panel, an additive effect of sensitivity was obtained with 93% sensitivity and approximately 80% specificity to distinguish early HCC from non-HCC tissue (FIGURE 1).

In addition, while most cases of HCC are associated with known etiologies, it would be ideal to have a single panel that can provide sufficient sensitivity and specificity for HCC screening, regardless of etiology. However, it may be necessary to construct etiologically-specific panels to improve the performance of markers. Recent developments in NGS have enabled the genome-wide sequencing studies of liver cancers, which cover mutational signatures with distinctive etiological backgrounds [13]. For instance, a significant correlation has been reported between somatic substitution patterns and habitual alcohol drinking [19]. Additionally, two previously suggested copy number variations at 13q12.11 and 11q11 of non-alcoholic fatty liver disease were also identified by the genome wide study approach [71–74]. Whether these risk markers are risk markers for non-alcoholic fatty liver disease-associated HCC needs to be studied.

In the next section, we briefly summarize HCC-associated cancer pathways, including whether or not there is a significant impact due to known etiologies, potential DNA modifications detected in tissue and most importantly, if these modifications have been detected in peripheral body fluids, which would serve as the foundation for the assembly of a single sensitive panel or etiologically specific panels for HCC screening.

HCC-associated cancer pathways & candidate DNA biomarkers for HCC detection

The multistep sequences of genetic and epigenetic alterations in liver cancer pathogenesis disrupt core cellular processes, such as cell cycle control, apoptosis and genome maintenance. The advent of NGS has allowed detailed mapping of the liver cancer genome, revealing extensive molecular alterations. Unlike some solid tumors, such as breast, lung or colon, development of HCC lacks a clear correlation to an oncogene. However, highly recurrent somatic mutations or methylations are found in the genes involved in some major cancer pathways, such as *TP53* and Wnt/ β -catenin pathways. This echoes the need for multiple markers to achieve a sufficient sensitivity for HCC screening. Here, we briefly review the key pathways and the DNA alterations, both genetic mutations and epigenetic DNA methylation, which have been reported in tissue as candidate DNA markers for building a panel of markers for molecular diagnosis of HCC. For this purpose, TABLE 1 lists the DNA alterations, both genetic and epigenetic, which are found in at least 5% of HCC tissue and have been reported by at least two independent studies, and these alterations are listed in order of incidence within each pathway. Also noted is whether or not each modification was previously detected in circulation.

Wnt/ β -catenin signaling pathway

The Wnt pathway is the most frequently altered pathway found in HCC, and activation of which leads to the nuclear translocation of the β -catenin and initiates cell proliferation.

Genes that are commonly altered in this pathway that are also detected in HCC are *APC*, *CTNNB1*, *AXIN1* and Secreted Frizzled related protein 1 (*SFRP1*) genes. At least 75% of HCC was found to contain DNA modifications in one of these genes [11]. Among these alterations, aberrant DNA methylation in the promoter region of the *APC* gene is the most frequent alteration found in HCC for approximately 50–80% of cases [75–78]. Genetic mutations of the *APC* genes have also been reported, but at a lower frequency (1.6%) [18]. The *mAPC* gene has been detected in the circulation of patients with HCC [38,79–82].

The next most frequently detected DNA marker is the epigenetic alteration of the *SFRP1* gene. *SFRP1* protein forms an inhibitory complex with the frizzled receptor and acts as a Wnt pathway inhibitor. It is also known to be a negative regulator of cell invasion and could possibly predict the metastatic potential of a tumor [83]. Hypermethylation of the *SFRP1* promoter is observed in more than 50% of HCC [27,84–86] and loss of heterozygosity is reported to be seen in 17.4% of cases [84].

As for genetic mutations in the Wnt pathway, the only frequent mutation also found in HCC is in the *CTNNB1* gene. Both point mutations and deletions were identified at the phosphorylation site in the region from codon 32 to codon 451 of exon 3 of the gene, accounting for 30–35% of HCC [11,18,67,87–94]. Interestingly, it was found that downregulation of the *SFRP1* gene acted synergistically with the mutation of the *CTNNB1* gene, effectively causing an upregulation of Wnt signaling in liver cancer [86]. Additionally, it was recently discovered via NGS analysis that mutations in the *AXIN1* gene, a downstream protein in the pathway, were found in approximately 15% of HCC [12,18,19].

The cell cycle control pathways

The second most altered pathway found in HCC tissue is the cell cycle regulation pathway. A frequently altered gene of this pathway is the *RASSF1A* gene, which is a member of the *Ras*-association domain family of genes that can associate with the *Ras* family of GTPases to regulate the cell cycle and trigger apoptosis [95]. It can also regulate microtubule dynamics and serve as a scaffold for multiple tumor suppressor complexes [96–100]. Abnormal signaling in *Ras* oncogenes was found in approximately 70% of all cancers [56,80,100]. Another frequently identified DNA modification in this pathway is methylation of *CDKN2A* (p16), which occurs in 50–70% of HCC. In addition to DNA methylation, mutations of the gene were also found, albeit less frequently (5–10%) [18,101,102]. Also within this pathway, mutations of the *TP53* gene, which are associated with chromosomal instability, occur in 20–50% of HCC. *TP53* mutations in codon 249 occur frequently (up to 50%) in aflatoxin-exposed populations [59,103,104]. Furthermore, *IRF2*, a gene upstream of *TP53*, was recently identified to be mutated in approximately 5% of HCC [18]. Among these, *mRASSF1A* and *TP53* mutations were detected in the serum and urine of HCC patients at rates up to 90 and 50% respectively, suggesting these genes could be promising biomarkers for non-invasive HCC screening [38,42,60,80,86,105–107].

Telomere maintenance

Loss of telomere is one of the hallmarks of cancer. Nault *et al.* recently described frequent mutations (59% in HCC and 25% in cirrhosis) in the promoter region of the telomerase

reverse transcriptase (*TERT*) gene [33]. Preferential integration of the HBV genome in the regions near or in the *TERT* gene has also been reported in five of the six HBV-HCC NGS studies [16,19,108–110]. Interestingly, higher rates of mutations in *TERT* (46% in HCV-HCC vs 16% in HBV-HCC) [111] and *CTNNB1* (62.5% in HCV-HCC vs 37.5% in non-HCV-HCC) [15] were reported in HCV-associated HCC. Recently, it was suggested that *TERT* is the gate keeper of HCC [112].

The chromatin remodeling pathway

Approximately 50% of HCC cases were found to have mutations in the chromatin remodeling pathway [19]. Among the mutations detected, *ARID1A*, *ARID1B* and *ARID2* are the most frequently detected, all of which are part of the SWI/SNF related chromatin-remodeling complex. It has been shown that siRNA targeting *ARID1A*, *ARID1B* and *ARID2* genes in HCC cell lines resulted in increased cell proliferation, suggesting their role as drivers of hepatocarcinogenesis [113]. *ARID1A* mutations are linked to HCC with alcoholic liver disease and overlap with tumors harboring *CTNNB1* mutations. *ARID2* mutations were associated with HCV-HCC [18]. Additional mutations, such as in the SMARCA and MLL families [18], and copy number losses, such as in *BRG* and *BRM* [114], have also been identified in this pathway associated with HCC, but have not been recurrent across multiple studies. Since these mutations were identified recently, no report as of yet shows the detection of these mutations in the periphery.

The mTOR pathway

The PI3K/AKT/mTOR signaling pathway, which plays an important regulatory role in cell proliferation, migration, survival and angiogenesis, is frequently dysregulated in HCC [115,116]. Elevated Akt phosphorylation is seen in 15–41% of HCC. However, the pathway activation is predominantly the result of ligand-based activation due to growth factor and growth factor receptor overexpression in HCC cells, and this is usually mediated by copy number alterations of growth factors or receptors. The most frequent recurrent modifications identified from this pathway are *PTEN* mutations and methylation [18,117,118]. In addition, *RPS6KA3* mutation was recently reported in about 10% of cases [11,18].

The JAK/STAT pathway

Genomic alterations in the *JAK/STAT* pathway have been reported in 45.5% of the HBV-HCC tumors, which are mainly caused by the aberrant methylation of the *SOCS1* gene (50–90%) [14,119,120]. This modification is consistent with the notion that activation of the JAK-STAT pathway could be a driving factor in HBV-associated HCC [14]. In addition to DNA methylation, the activating mutations of *JAK1* were also found, however, these modifications occurred less frequently, and were identified in only approximately 9% of HCC [14].

Other pathways

In addition to disrupting core cellular pathways, the aberrant methylation of one of the detoxification genes, *GSTP1*, and a protease inhibitor, *TFPII2*, were identified in a significant portion of HCC (~50% for both genes) [80,121–125]. Since these genes are

distinct from the major pathways discussed, they should also be included for added value when a sensitive HCC DNA marker panel is assembled. Other pathways such as the oxidative stress pathway/NRF2-KEAP1 and the MLL pathway have been reported with several infrequent modifications to be involved in HCC pathogenesis [15,29], thus they are not included in this discussion.

Identification of AFP-negative HCC by DNA markers

As discussed earlier, the current 'gold standard' serum marker, AFP, and its fucosylated glycoform, L3, are of limited value because they have sensitivities of only 25–50% for early HCC [126]. Moreover, there is currently no biochemical marker that can detect AFP-negative HCC, in which the serum AFP level is <20 ng/ml, as suggested by the American Association of Liver Diseases [126]. Because of this, it was of interest to see whether DNA biomarkers could detect AFP negative HCC. DNA marker distribution was plotted for the HCC tissue from patients with serum AFP values available for analysis, as shown in **FIGURE 2**. In this study population (n = 112), 61 patients (54%, 61/112) with HCC had AFP serum levels <20 ng/ml, and their HCC samples were therefore considered to be AFP-negative. Encouragingly, DNA markers were found in 92% (56/61) of the AFP-negative HCC tissues, suggesting the potential of these markers to be used to detect AFP-negative HCC, should they be detectable in the periphery.

Specificity of HCC DNA markers for cancer screening & early detection

More than 80% of HCC cases arise from cirrhotic liver [127]. The process of carcinogenesis is a continuous process and is believed to occur over a course of years, initiating from a cirrhotic liver and evolving into the size of a tumor that can be detected via ultrasound imaging, which is approximately 2 cm in diameter [128]. The DNA mutations and aberrant epigenetic DNA methylation patterns that drive carcinogenesis, the drivers, are expected to exist at the onset of carcinogenesis, thus, occur mostly in cirrhotic liver. Many, if not all, of these early markers were also detected in adjacent non-HCC tissue, which, as part of the cancer microenvironment, is the field where HCC nodules are expected to arise. DNA modifications of these drivers would be good candidates as DNA markers for the early detection. However, these markers would not be very specific to HCC, as the observed modifications could already exist in cirrhotic liver or pre neoplastic nodules. As for HCC screening or early detection, these early markers are important to identify subjects for more sophisticated imaging diagnosis.

As discussed above, one approach to potentially improve the specificity of the test for screening and early detection is to combine early, organ-specific, and highly-specific late markers into a panel evaluated with a trained algorithm. Another approach to improve the specificity of the marker is to detect the region of the gene that is more specific for HCC. This is of particular importance for methylated DNA markers as there is emerging evidence suggesting that the specificity of methylation of different regions of the CpG island of the tumor suppressor genes could vary the performance of biomarkers for cancer detection, or in its association with clinicopathology [75,121,129,130]. For instance, we noticed a variation between studies of the specificity for distinguishing tumor from non-tumor tissues of three

extensively studied HCC-associated aberrantly methylated DNA markers, *mAPC* [75], *mGSTP1* [121] and *mRASSF1A* (manuscript submitted). After performing a comprehensive analysis of the DNA methylation profile of these three genes, using bisulfite-specific (BS)-PCR sequencing and methylation-specific PCR assays, comparing tissue DNA from normal-liver, hepatitis, cirrhosis, HCC and a dozen non liver, normal tissue samples, we demonstrated that little specificity for HCC was found when *mGSTP1* was analyzed at the 3' region of the -48-nt position and when *mAPC* was analyzed on the antisense strand of the DNA. Conversely, a high specificity was obtained when *mGSTP1* at the 5' region of the 48 nt position and the sense strand of DNA of the *mAPC* gene were examined [75,121]. Furthermore, among three previously defined promoter regions of *RASSF1A*, P1, P2 and E1, we found that methylation of the P1 region was more specific to HCC as compared with P2 and E1 (data not shown). Collectively, previous studies suggest that the choice of the promoter region to examine DNA methylation is a critical step that should be carefully considered in the development of DNA methylation markers for cancer screening [129].

Detection of HCC DNA markers in peripheral body fluids

As previously discussed, DNA markers can only be useful for cancer screening or early detection if the marker can be detected in the periphery. **TABLE 1** lists major genetic and epigenetic DNA modifications that have been identified to have an association with HCC as compared with hepatitis and cirrhosis. There have been many attempts to evaluate the performance of each marker separately, or in combination with other markers, in the periphery. We have summarized these data in **TABLE 2**, which lists the markers that have been reported to be found in the body fluid and the performance of each marker for HCC screening. Please note, the ranges of the sensitivities and specificities listed are the collection of the results from references. Note, these values of sensitivity and specificity reported by each study were dependent on the cutoff value of the marker used in the study, and when the sensitivity was reported to be low, it was often associated with a high specificity. Moreover, the difference of the study populations and sizes of the studies could also impact the marker performance which is one of many reasons why 99% of biomarkers failed to be translated into clinic.

Among the genetic mutations that have been associated with HCC, mutation of *TP53* is the only mutation reported in the circulation of HCC subjects. However, *TP53* mutations were detected in the circulation of patients with HCC with an excellent concordance [103,106,131–133]. We have further shown that the *TP53* 249T mutation can be successfully detected in the urine from approximately 50% of HCC patients [42].

Contrary to genetic mutations, almost all frequently methylated genes in HCC have been reported to be detectable in the circulation of patients with liver cancer with a good concordance between tissue and serum/plasma specimens, underscoring the potential of these methylated DNA markers for HCC screening [82,105,134–136].

As mentioned above, multiple markers are needed to obtain a sufficient sensitivity for screening. Several recent studies have investigated the performance of various combinations of methylated markers in circulation for HCC screening [80,81,119,137] with promising

results. For instance, Huang *et al.* performed quantitative analysis of a circulating methylated DNA marker panel (*APC*, *RASSF1A*, *GSTP1* and *SFRP1*) in a study of 150 patients with and without HCC, which demonstrated a significantly better performance of the panel (92.7% sensitivity, 81.9% specificity with area under the curve of 0.933), as opposed to individual markers [80]. Recent advancements of NGS have allowed the comprehensive comparison of DNA alterations between tumor and non tumor in the circulation [138-141]. Thus, the number of potential markers used to distinguish HCC from non-HCC has greatly progressed to hundreds or even thousands. However, to be the most effective, the development of an algorithm to be used with these markers will be necessary in order to process the large amount of data that would be produced. Overall, this approach provides an extremely powerful tool in detecting tumor derived mutations and copy number aberrations for not only cancer detection and monitoring, but also for providing cancer genetics for better disease management.

HBV-based viral DNA markers

Chronic HBV infection is a major risk factor for developing HCC, and it is associated with over 50% of HCC cases world-wide and up to 70–80% of HCC cases in HBV-endemic areas. The contribution of HBV infection to the pathogenesis of HCC is believed to be multifactorial, further compounding this risk factor. During carcinogenesis of HBV-related HCC, it is believed that modifications of the HBV genome, both genetic and epigenetic, occur not only in the host genome, but also in the viral genome. Although detection of HBV-DNA markers will only be useful for patients with HBV-related infections, the inclusion of HCC associated HBV-DNA markers provides potential screening markers that are liver specific. The most commonly studied HCC associated HBV mutation is the basal core promoter double mutation A1762T/G1764A [142–149]. This mutation causes an increase in the host immune response and diminishes HBeAg production by suppressing transcription of precore mRNA [143]. It has also been suggested to be an early marker event during carcinogenesis, as detection can occur up to 10 years before HCC diagnosis [150,151]. Furthermore, it is a particularly useful marker in a specific subset of male patients who are HBsAg+ [152]. This double mutation marker has been detected in both the circulation and the trans renal DNA of patients with HBV-HCC [153], lending itself to potentially be a good marker for screening or risk stratification of HBV-HCC patients. This mutation has also been implicated in HCC-risk prediction in HBV genotype B or C patients [143,154,155].

The preS region of the HBV genome plays a role in the viral interaction with the host immune response due to the B-cell and T-cell epitopes in this region [142,156]. PreS deletions will decrease the expression of surface proteins and may contribute to immune escape. Studies have linked progressive liver disease with a higher frequency of preS deletions [157–159]. Particularly, the 5' terminal (nt. 3206–60) of the preS region was found to be the most frequently deleted portion in HCC, compared to non-HCC patients (43 vs 28%) [142]. Also, preS1 and preS2 promoter mutations have been reported to be significantly higher in HCC patients (19.7 vs 3% and 15.3 vs 8.9%, respectively) [142]. Additionally, a precore mutation, G1899A, has been found to be significantly associated with HCC in Thailand [160,161].

The performance of these mutations in predicting HCC varies in such a way that no single genetic mutation is able to sufficiently identify all HBV-related HCC. In recent studies, when at least six of eight particular HBV mutations (G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A and G1899A), are used in combination, the panel has a predictive power of 94.3% with a specificity of 97.3%, although the sensitivity was only 44% ($n = 75$) [162,163]. While these studies looked at only HBV infected patients with genotype C2, the area under the curve of mutation number versus AFP was comparable (0.825 vs 0.869).

There have been increasing studies in determining both mutation frequency and mutation correlation with HCC in HBV patients with genotype D, as prior studies are often only applicable for those HBV patients with genotypes B or C [164,165]. These emerging studies may shed light on new mutations associated with HCC that are specific for HBV genotype D patients. Mutations in HBV genotype D patients who have been found to be associated with HCC are G1727 (5 vs 35%), C1741 (7.5 vs 30%), C1761 (2.5 vs 15%) and T1773 (52.5 vs 95%) (non HCC, $n = 107$ vs HCC, $n = 45$) [164].

Lastly, there have been emerging studies looking at epigenetic modifications in the HBV genome. The HBV genome has 2–3 conventional CpG islands depending on the genotype [166,167]. Interestingly, these CpG islands are found at strategic locations in the regulatory elements of the HBV genome [168]. To better understand the methylation status of HBV DNA in hepatitis, cirrhosis and HCC patients, a comprehensive methylation profile of three CpG islands in the HBV genome was assessed via BS sequencing. From which, the methylation pattern of each of the three CpG islands was compared by using BS specific PCR and methylation-specific PCR assays. In a sample size of 94 patients (hepatitis, $n = 11$, cirrhosis, $n = 9$ and HCC, $n = 74$), we observed that the methylation of CpG Island 1 and 3 of HBV DNA from liver tissue was significantly higher in HCC, as compared with hepatitis and cirrhosis (manuscript in preparation). Furthermore, the extent of the HBV genome methylation was significantly correlated to the disease progression of the infected liver ($p < 0.05$ by student's *t*-test). While these results are promising, co-relation of HBV genome methylation and HCC carcinogenesis in the periphery warrants further research to explore its potential as a cancer biomarker.

Challenges for the development of biomarkers for clinical applications

The development of a biomarker is a process that begins with biomarker discovery and is concluded with the evaluation of the impact of the biomarker on a clinical outcome. Unfortunately, only 1%, possibly less, of published cancer biomarkers actually enter clinical practice; most published biomarkers have failed. To facilitate biomarker development, supportive platforms have been established by NIH that offers to improve the development process, including the creation of the Early Detection Research Network. As a result, a five phase guideline for cancer screening biomarker development has been generated [169], and standards have been suggested for study designs of biomarker evaluation for either classification or prediction of the outcome of a specific cancer [170]. Instructive anecdotes relating specifically to cancer biomarkers have been collected, and the commentary has been published [171–173]. With the application of NGS in the cancer genome, the number of

DNA modifications for HCC increase exponentially. Therefore, more attention is necessary so as to avoid developing bio markers for modifications that lead to failed biomarker development for either molecular diagnostics or as targets for liver cancer therapy.

Expert commentary

All cancers, including HCC, result from accumulation of genomic and epigenomic modifications. Identification of DNA modifications (markers) underlying the development of HCC should permit an unambiguous diagnosis and provide genetic characterization of the tumor to tailor a more precise treatment plan. Thus, DNA-based markers can serve not only as a first-line test for screening and diagnosis of high-risk individuals, but can also play important roles in the clinical management of HCC, such as prognosis prediction and selection of targeted therapies [174]. Given the high heterogeneity of HCC, different combinations of cancer pathways could be altered in a manner dependent on patient etiologies, and therefore a multi-gene panel approach will be needed to obtain a high sensitivity in this DNA marker based screening/surveillance test. Genetic and epigenetic alterations are two unique facets of DNA damage that go hand in hand toward contributing to tumor formation. Thus, an ideal test should utilize a technology capable of detecting both genetic and epigenetic DNA modifications. While genome wide sequencing and methylation profiling are best suitable for discovery of the alterations of the drivers responsible for tumor initiation, a targeted sequencing approach is more applicable for screening and diagnostics due to a greater cost-effectiveness and easier data management. An example of this targeted NGS approach has been applied in a breast cancer study using plasma DNA [175]. In order to optimize this approach for molecular diagnostics, it will be critical to develop well-defined algorithms that can analyze DNA marker profiles with etiologies and other risk factors, including age, gender and liver function, which will be needed in order to translate the wealth of knowledge for the DNA markers obtained from the advent of NGS technology.

Five-year view

In the near future, we anticipate that a large amount of NGS data, of both genetic and epigenetic DNA modifications, comparing HCC to non-HCC in tissue of distinct etiologies, as well as in the periphery (plasma and urine), will become available from an adequately powered sample size to provide the genetic/epigenetic landscape of HCC, while comparing various etiological factors. This will allow the identification of driver mutations across the spectrum of liver emerging from these very diverse etiological factors. The abundant DNA biomarker information obtained from tissue studies will allow molecular characterization of HCC, once the tumor is confirmed via biopsy or removed through surgery, to tailor the treatment plan. Given the recent breakthroughs in HCV treatment, we envision the incidence of HCC will be reduced and the reduction of this risk factor might also result in a decrease in the overall incidence of HCV related DNA modifications in HCC. NGS data from DNA bio markers detected in the periphery will allow for strategic designs of a marker panel for screening and diagnosis. Such a liquid biopsy will provide the molecular characterization of a tumor, in order to enable improved disease treatment plans and to monitor for recurrence. We envision a panel of genetic and epigenetic DNA markers with other clinical variables such as patient risk factors (age, gender, hepatitis B/C, alcohol, NASH, etc.), clinical

laboratory information (AFP, liver function) and radiographic studies (ultrasound), to be integrated into an algorithm for both screening and early detection. The continuous advances in sequencing technology will lower costs, making it possible to use DNA sequencing as a routine screening or surveillance tool. Simultaneously, efforts will be made for the standardization and quality control of clinical NGS. We also anticipate the validation of surveillance values of these HCC DNA biomarkers with prospective cohort studies for their translation into actual clinical use.

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•• of considerable interest

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Key issues

- The current methods of surveillance are inadequate and there is an urgent need for better methods.
- A non-invasive sensitive approach is critical for surveillance, given the chronic nature of the disease and well-defined high-risk populations.
- Due to hepatocellular carcinoma (HCC) heterogeneity, a panel of multiple DNA biomarkers is required for a highly efficacious screening test.
- DNA biomarkers are promising screening tools for AFP-negative HCC.
- A comprehensive algorithm integrating data from targeted NGS, various risk factors and laboratory tests could be a very powerful tool, not only for HCC screening and molecular diagnosis, but also for personalized management of patients.

Figure 1. Multi-marker receiver operating curves for *mAPC*, *mGSTP1*, *mRASSF1A* and *TP53 249T* mutation individually and in combination for distinguishing hepatocellular carcinoma (n = 120) from cirrhosis (n = 35) and hepatitis (n = 35) tissues

The area under the ROC is indicated in parentheses following each marker.

HCC: Hepatocellular carcinoma; *mAPC*: Methylated *APC*; *mGSTP1*: Methylated glutathione S-transferase P1; *mRASSF1A*: Methylated *RASSF1A*.

Figure 2. Detection of four DNA markers, mAPC, mGSTP1, mRASSF1A and TP53 249T mutation in hepatocellular carcinoma tissues (n = 112)

Marker distribution in individual HCC with available AFP value in the study population.

Filled boxes represent a marker detected or positive.

Empty boxes represent a marker not detected or negative by cutoff.

AFP: α -Fetoprotein; HCC: Hepatocellular carcinoma; *mAPC*: Methylated *APC*; *mGSTP1*: Methylated glutathione *S*-transferase P1; *mRASSF1A*: Methylated *RASSF1A*.

Table 1

Frequently reported genetic mutations (G) and epigenetic aberrant DNA methylations (M) in hepatocellular carcinoma tissues.

Pathway	Gene	DNA alteration	Incidence (%)	Reported in circulation (Yes/No)	Ref.
Wnt signaling	<i>APC</i>	M/G	80–93/1–2	Yes	[11,18,38,75–80,82,176]
	<i>SFRP1</i>	M	42–63	Yes	[27,80,83–86,119]
	<i>CTNNB1</i>	G	19–33	No	[11,18,87–94,177]
	<i>AXIN1</i>	G	13–15	No	[11,12,18]
Cell cycle/apoptosis	<i>RASSF1A</i>	M	55–85	Yes	[80,120,178–180]
	<i>CDKN2A</i>	M/G	50–70/5–10	Yes	[17,18,81,101,102,120,176,181]
	<i>TP53</i>	G	20–50	Yes	[11,18,60,87,182–185]
Telomere maintenance	<i>TERT</i>	G	29–59	No	[33,111]
mTOR signaling	<i>PTEN</i>	M/G	16–43/4–16	No	[18,117,118]
Chromatin remodeling	<i>ARID1A</i>	G	10–20	No	[11,18,19]
	<i>ARID1B</i>	G	5–10	No	[11,18,19]
	<i>ARID2</i>	G	5–18	No	[11,18,113]
JAK/STAT	<i>SOCS1</i>	M	50–93	No	[14,119,120]
	<i>JAK1</i>	G	9	No	[14,186]
Detoxification	<i>GSTP1</i>	M	38–80	Yes	[80,121,122]
Protease inhibitor	<i>TFPI2</i>	M	47	Yes	[124,125]

Table 2

Hepatocellular carcinoma DNA markers detected in circulation.

Pathway	Marker	Sensitivity (%)	Specificity (%)	Periphery	Cases/controls [†]	Ref.
Wnt signaling	<i>mAPC</i>	24–68	88–97	Plasma	26/16, 72/41, 108/60, 28/0	[38,80–82]
		100	100	Serum	23/8	[79]
	<i>mSFRP1</i>	28.7–55.6	87.8	Plasma	72/41, 108/60	[80,81]
Cell cycle/ apoptosis	<i>TP53 249T mutation</i>	15–47	46–86	Plasma	39/0, 186/98, 14/5, 20/10, 53/53, 89/131, 84/56, 25/20, 55/52, 198/325, 176/133, 41/74	[39,60,61,103,132,133,187–194]
		4–18	83.3	Serum	76/110, 158/0, 108/0	[106,195,196]
		53	75	Urine	17/15	[42]
	<i>mCDKN2A</i>	19–59	68–100	Plasma	26/16, 39/0, 28/0	[38,39,82]
		44–92	68–96	Serum	50/50, 25/17,66/43	[197–199]
mTOR signaling	<i>mRASSF1A</i>	27–94	64–100	Plasma	26/16, 72/41, 40/10	[38,80,200]
		70–100	52–100	Serum	35/10, 50/50	[179,197]
Detoxification	<i>mGSTP1</i>	19–55	90–93	Plasma	26/16, 72/41	[38,80]
		50	62.5	Serum	32/8	[136]
Protease inhibitor	<i>mTFPI2</i>	47	80	Serum	28/0, 43/26	[82,125]
Cell adhesion	<i>mCDH1</i>	17–27	75–97	Plasma	43/26	[125]

mAPC: Methylated APC; mGSTP1: Methylated glutathione S-transferase P1; mRASSF1A: Methylated RASSF1A; mSFRP1: Secreted Frizzled-related protein 1.

[†]Number of cases and controls used in each study are listed corresponding to the order of the references listed in this table.