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Review Article

The potential of circulating tumor DNA to use as a molecular marker to screen and diagnose hepatocellular carcinoma: A systematic review

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ABSTRACT

Now a day's molecular characterization of individual patients' tumor cells is becoming instantly important for early detection and effective treatment of the disease. The idea of applying liquid biopsy technologies for early diagnosis of cancer through the specific and sensitive determination of circulating tumor DNA (ctDNA) among circulating free DNA (cfDNA) in plasma is a relatively recent approach with considerable promise, but also presented with great challenges. Ongoing advancement in the field has shown that ctDNA has huge potential to serve as a biomarker for early detection and precision treatment as well as prognosis of hepatocellular carcinoma (HCC). As ctDNA in HCC patients harbors the molecular characteristics of HCC tumor cells, ctDNA analysis in the blood of HCC patients might be an adequate and non-intrusive approach for locating tumors, disease prediction, and treatment. In the sight of this fact, this review tried to sum up and discuss the surveillance of HCC, the origins and molecular characteristics of molecular markers of hepatocellular carcinoma, the current status, and the potentials of ctDNA as a marker for HCC surveillance and early detection. Moreover, this review also describes the major tumor-specific genetic modifications in ctDNA, such as DNA methylation, microsatellite alterations, point mutations, chromosomal rearrangements. Finally, the challenges associated with the clinical use of ctDNA for HCC detection are also discussed.

1. Introduction

Hepatocellular carcinoma (HCC) is the primary malignancy of the liver that occurs predominantly in chronic liver disease and cirrhosis patients.[1] Its origins are believed to be the hepatic stem cells although this remains the subject of investigation.[2] Liver diseases, such as viral hepatitis (hepatitis B and C virus) infection, autoimmune hepatitis, hereditary hemochromatosis, alcohol-related liver disease, non-alcoholic fatty liver disease (NAFLD), and Wilson's disease are associated with an increased risk for the development of liver cirrhosis and HCC.[3,4] This multidimensional nature of the disease has contributed to the complexity of tumor study. The majority of patients who develop HCC in the West have underlying cirrhosis primarily due to the hepatitis C virus (HCV), alcohol, and nonalcoholic steatohepatitis.[5]

Globally, HCC is the fifth most common cancer in men and the seventh in women, with more than 748,000 new cases annually, accounting for 9.2% of all new global cancer cases (7.9% in men; 3.7% in women).[6] This also accounted for 90% of all cases of primary liver cancer, with nearly 800,000 new cases annually.[7] While overall cancer mortality has decreased, liver cancer-related mortality has been rising steadily by 2.4% yearly.[8] This high mortality rate is mainly due to a lack of therapeutic options and difficulty in early detection and undermined prognosis.[9]

At present diagnosis of HCC is made by radiologic imaging techniques, such as computer tomography and magnetic resonance imaging even though histology remains the gold standard in inconclusive cases.[9] However, proper

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diagnosis of small HCC is the most difficult thing. Because imaging tests do not frequently present typical vascular patterns and acquisition of reliable histological specimens also often require repetitive biopsies and pathological interpretation.[10] Thus, the detection of premalignant lesions is the most challenging aspect of hepatology. However, in recent years ctDNAs, being a popular class of liquid biopsy biomarkers, are believed to be easily detected in the plasma of cancer patients even in the early stages of the disease.[11] This is because cDNAs display considerable variations in DNA sequences.[12]

2. Molecular markers used for the diagnosis of hepatocellular carcinoma

Like other cancers, HCC is a disease of the genome, caused by genetic and epigenetic DNA modifications.[13] Thus, identification of DNA modifications (mutation or methylation) underlies the development of HCC which permits an unambiguous diagnosis of HCC cases. The identification of DNA modifications associating with HCC tissues is achieved due to the introduction of next-generation sequencing (NGS). However, in whatever way, these candidate DNA markers will not be useful for either diagnosis or surveillance unless they can be detected in the peripherals, without liver biopsy. ctDNA and cfDNA are the well-known DNA markers that can be used for the diagnosis of HCC without a liver biopsy. cfDNA is defined as extracellular DNA present in plasma or serum samples[14] which can be detected not only in patients suffering from cancer or other diseases but also in healthy individuals.

3. The Historical overview of ctDNA

Extracellular or cell-free nucleic acids (DNA and RNA) have been distinguished in several body fluids, such as blood, urine, stools, milk, bronchial lavages, or ascites.[15] ctDNA was first found in plasma tests by Mandel and Metais[16] and, just numerous years after the fact was verified by Stroun and his colleagues[17] as the ctDNA in cancer patients' plasma is originated from tumor cells. Specialized advances in the recognition and evaluation of RNA and DNA have broadened the potential application of RNA and DNA for molecular diagnosis and monitoring of diseases. Particularly, ctDNA was found to convey tumor-related hereditary modifications and consequently, it has been considered as a potential malignancy indicative marker for a non-obtrusive test.[18]

In 1977, researchers made a new observation that cancer patients carried cfDNA in their peripheral blood.[19] Initially, the development of further characterization of cfDNA was slow due to the technological limitations of the pre-genome era. However, the technological advancement in the genomic era after two decades researchers proved unambiguously that this species of nucleic acid was derived from tumor tissues by the presence of characteristic cancer mutations.[20] Indeed, extensive progress was not made until the recent advent of NGS technology in combination with the early findings of Cytosine Guanine Phosphate (CGP), which significantly improved the sensitivity and specificity of ctDNA detection.[21] Subsequently, research in this field has entered a "golden age" in which the huge potential of ctDNA investigations in tumor diagnosis and treatment is becoming ever clear.[22]

4. Origins and Molecular Characteristics of ctDNA

The term cfDNA encompasses all kinds of extracellular DNA molecules, such as genomic DNA, mitochondrial host DNA, and foreign viral or bacterial DNA found in serum or plasma and other body fluids of vertebrates.[23] ctDNA is a type of serum cfDNA found in patients with cancer and contains abundant information regarding tumor characteristics, highlighting its potential diagnostic value in the clinical setting.[24] Early studies showed that ctDNA possessed many cancer-associated molecular characteristics, such as single-nucleotide mutations,[25] methylation changes,[26] and cancer-derived viral sequences which are considered to be derived from tumor tissue. As a result, these findings are significant for the development of future ctDNA detection technology.[27]

cfDNA is released into circulation by various pathologic and normal physiologic mechanisms, such as shedding of DNA into the bloodstream from dying cells during cellular turnover or apoptotic and necrotic cells.[28] Even though cfDNA are originated from cell death, live cells can also spontaneously release newly produced DNA as part of a homeostatically regulated system. For instance stimulation of lymphocytes can lead to the release of large amounts of cfDNA in the absence of cell death[17] which means that cfDNA of cancer patients can be derived from both non-malignant and malignant cells. Under normal physiologic conditions, apoptotic and necrotic cells are also cleared by infiltrating phagocytes and cfDNA levels are relatively low.[29] However, this mechanism does not act effectively in the tumor mass. Most cfDNA fragments are measured between 180 and 200 base pairs (bp), suggesting apoptosis as the predominant source of cfDNA in the circulation.[30] In solid tumors, cfDNA can be also released through necrosis, autophagy, and other physiologic events induced by microenvironmental stress and treatment pressure. In contrast to apoptosis, necrosis generates larger DNA fragments due to incomplete and random digestion of genomic DNA.[31]

In cancer patients, a fraction of cfDNA is tumor-derived and is termed as circulating tumor DNA (ctDNA). Cancer patients have much higher levels of ctDNA than healthy individuals, but the levels differ widely based on the patient's tumor burden, stage, vascularity, cellular turnover, and response to therapy.[32] Interestingly, the

percentage of circulating DNA derived from cancer cells ranges from 3% to as much as 93%.[33] Since cancer cells die via multiple mechanisms that lead to DNA cleavages, such as apoptosis, necrosis, and autophagy, ctDNA displays less uniformity in size and integrity relative to cfDNA in healthy individuals.[34] Apoptotic cells shed DNA fragments approximately 185 to 200 bp in length.[30] Some researcher groups report that ctDNA has less integrity and is smaller relative to cfDNA while others report the opposite.[35] More recently, one study showed that decreased cfDNA integrity and increased cfDNA concentrations distinguish normal individuals from patients with primary and metastatic breast cancer.[36] Another study that used single-base pair resolution sequencing clearly showed that the shorter DNA fragments in cancer patients' plasma preferentially contained tumor-associated copy number alterations which suggest that the isolation and analysis of short DNA fragments may improve the sensitivity of ctDNA detection.[33]

5. The current status of ctDNA for the diagnosis of hepatocellular carcinoma

In current clinical practice, genotyping is achieved using DNA obtained from a tissue biopsy.[22] However, tissue biopsy can only obtain local and static tumor information and is unable to reflect the real-time tumor genotype due to heterogeneity and constant evolution of tumors.[37] This problem can be overcome via ctDNA analysis since it can reflect the genetic mutations of the whole tumor tissue.[38] Moreover, ctDNA from the same patient at different stages can be used to dynamically monitor the genetic mutations during cancer progression. Therefore, liquid biopsy-based on ctDNA analysis might improve tumor genotyping and targeted cancer therapy.[39]

In principle, ctDNA fragments contain genetic defects identical to those of tumor tissues, including point mutations, rearrangements, amplifications, microsatellite instability (MSI), loss of heterozygosity (LOH), and tumor-associated DNA methylation.[40] Therefore, the detection of such genetic modification via blood-based tumor genotyping assays using ctDNA will be greatly beneficial for guiding personalized cancer treatment.[41]

5.1. The potentials of ctDNA as a marker for HCC surveillance and early detection

Malignant transformation of hepatocytes occurs when DNA alterations either activated or inactivated certain cancer pathways that induce uncontrolled growth of the cells, or clonal expansion.[42] The development of HCC, like 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.[43] Thus, identification of the DNA modifications underlying the development of HCC should permit an 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,[44] and several candidate cancer biomarkers have been exploded since the introduction of genome-wide sequencing of diseased tissue DNA using NGS and genome-wide methylation study using methylation arrays. 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.[45]

DNA containing cancer 'signatures' (mutations or hypermethylation) has been found in the plasma, serum, and urine of cancer patients, including in HCC patients. These tumor-associated DNA modifications in the plasma or urine were consistent with those modifications detected in the primary tumor demonstrating that tumor-derived DNA can be detected in the circulation via blood or urine if a tumor is present.[46]

Several studies have addressed the possible mechanisms of the presence of ctDNA in the circulation.[47,48] Briefly, this ctDNA could be either from the primary tumor or from circulating tumor cells due to cell death by either necrosis or apoptosis, or even micro vesicle-released DNA in the circulation originating from tumor cells.[49] Regardless of the various sources of ctDNA, the presence of ctDNA in circulation provides a great promise to use blood or urine as a liquid biopsy to profile cancer genetics for cancer screening and early detection.[50]

5.2. CtDNA of hepatocellular carcinoma

Among the different circulating cell-free DNA, ctDNA is one of the components of cfDNA that can be released into the circulation of cancer patients from tumor cells that undergo metabolic secretion, apoptosis, or necrosis.[51] In contrast to normal cfDNA, ctDNA carries tumor-specific genetic or epigenetic alterations, like point mutations, copy number variations, chromosomal rearrangements, and DNA methylation patterns.[52] Thus, the invasive examination of ctDNA with a small amount of peripheral blood from patients could show these genetic and epigenetic alterations related to particular cancer which offers a remarkable opportunity for monitoring tumor genomes in a non-invasive, convenient, and accurate manner.[22] Some studies have examined the feasibility of ctDNA in detecting early-stage cancers and fewer still have considered ctDNA for the screening of pre-symptomatic cancer patients.[53] However, other studies argue that effective screening of asymptomatic cancer patients needs a previous knowledge of mutations in genes; ctDNA is most suited for the detection of advanced cancers with well-known mutations.[47]

Table 1: Potential application of ctDNA in clinical oncology.[40]

Cancer screening	Localized cancer	Metastatic cancer	Refractory cancer
Early diagnosis and intervention	Identifying specific genomic alterations to guide therapeutic selection, monitoring tumor burden and therapeutic responses, detecting minimal residual disease, assessing risks of dissemination and recurrence	Early identification of relapse and treatment resistance, the guidance of treatment selection, and monitoring therapeutic responses	Understanding the mechanism of resistance, and determining new treatment

6. Major tumor-specific genetic modifications in ctDNA used for HCC detection

The cfDNA is discovered first by Mandel and Metais in 1948.[54] Later Leon et al[19] also found that cancer patients had a relatively higher level of cfDNA than healthy individuals**.** This finding exhibited the potential of cfDNA as a biomarker of cancer including HCC. In 1989, ctDNA was noted to be a fraction of cfDNA in the blood.[17] Moreover, ctDNA is found to harbors cancer-specific mutations, demonstrating the potential utility of ctDNA in clinical applications.[55] As a result, quantification and detection of tumor-specific mutations in ctDNA appear to be more relevant to study tumor progression.[56]

6.1. DNA Methylation

DNA methylation plays a crucial role in regulating gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA.[57] This process is often dysregulated in tumor cells.[58] Aberrations of DNA methylation in the gene promoter region or the non-coding genomic sequences are involved with tumor initiation, dissemination and metastasis establishment, and progression.[59] The status of DNA methylation is very stable, even in circulation; thus it can be assessed to monitor tumor-related processes. Aberrant DNA methylation has been first detected in the plasma and serum of lung,[60] breast,[61] and liver cancer patients in 1999.[62] Since then, many studies have indicated the potential of ctDNA methylation as a diagnostic and prognostic marker for cancer patients.[63]

Methylation alterations occur on many genes associated with the initiation and progression of HCC. Extensive studies have shown the alterations of DNA methylation in the promoter region of Glutathione S-transferaseP1 (GSTP1),[64] cyclic independent kinase inhibitor p15[65] and p16[66] of tumor tissues in HCC patient. Thus, successful detection of hypermethylated GSTP1,[67] p15,[62] and p16[68] in cfDNA from HCC patients may allow the development of a blood-based assay for HCC diagnosis. Methylation alterations of the RAS association domain family 1A (RASSF1A) were also detected in cfDNA of HCC patients.[69] Furthermore, hepatitis B virus (HBV) carriers undergoing surveillance and subsequently developing HCC had significantly higher levels of RASSF1A from the time of enrollment to cancer diagnosis.[70] Another gene with methylation abnormality that has been detected in HCC patients has long interspersed nucleotide elements (LINE-1) which are highly associated with the progression and invasiveness of HCC.[71] Thus, the assessment of circulating methylation on ctDNA may provide a promising tool in HCC diagnosis and management.[72] Unlike genetic alterations, such as mutations and deletions, epigenetic changes are also potentially reversible and can be restored to their normal state by epigenetic therapy which is promising and therapeutically relevant.[73] This makes the detection of methylation a better approach for screening of HCC using plasma or serum samples.[74]

6.2. Microsatellite alterations

Highly polymorphic DNA repeat regions are frequently present in the genomes.[75] Since the discovery of tumor-derived microsatellite alterations in cfDNA by Allison and co-authors[2] interests are growing to use cfDNA alterations as a marker. Loss and length alteration in these regions are common in a variety of cancers, which can be used as markers for cancer diagnosis. Comparative genomic hybridization (CGH) technique has enabled scientists to define some microsatellite alterations in HCC, such as chromosome 8p, 17p, and 19p deletions[76] and the loss on 8p and 19p, which might contribute to HCC metastasis.[77] In the early 20^{th} century, a microsatellite marker screening was performed in the primary tumor and serum sample in HCC patients[78] which provide early evidence for the clinical utility of this approach. Two microsatellite markers on chromosome 8p, D8S258, and D8S264, have also been determined as contributors to HCC metastasis by comparing primary tumors and matched metastases.[77] Interestingly, only an allelic imbalance at D8S258 was found in the cfDNA of HCC patients, and the combination of both the allelic imbalance and a higher level of cfDNA are found to be well correlated with the decrease in disease-free and overall survival rates.[76]

6.3. Point mutations

Tumor progression involves the accumulation of both inactivations of tumor suppressor genes and the activation

of proto-oncogenes.[79] Ser249 of TP53 is the most reported mutation hotspot in HCC patients, and mutation of this site leads to the deficiency in its specific DNA binding ability.[80] Recently, TP53 Ser249 mutant in plasma has been reported to be highly associated with cirrhosis and HCC in China and Africa, a region with high HBV prevalence and high Aflatoxin B1 exposure.[81] Interestingly, this mutation was also detected in noncancerous hepatic tissues of HCC in plasma DNA of a few healthy individuals, and patients with relatively more severe cirrhosis which indicate this mutation might be involved in the early development of HCC and accumulated during HCC progression.[82] However, since this mutation, like many other mutations, occurs in other types of cancers, it cannot be excluded that ctDNA harboring this mutation is released from other tissues.[83]

6.4. Chromosomal rearrangements

Chromosomal rearrangements are composed of structural variations, including deletions, insertions, inversions, duplications, translocations, and others.[84] Such genomic rearrangements associated with cancer have been determined in HCC tumors through whole-genome sequencing.[85] Therefore, detection of such chromosomal rearrangements in ctDNA with prior knowledge of specific DNA aberrations and recurrent "hot spot" mutations by sophisticated PCR-based methods is crucial for their detection in the cfDNA of cancer patients.[86] Since chromosomal rearrangements have demonstrated greater tumor specificity, it can be identified in tumor and subsequently in the plasma of patients using quantitative PCR.[87] and next-generation sequencing (NGS) approaches.[88]

7. Challenges associated with the clinical use of ctDNA for HCC detection

Even though tumor-specific mutations and methylations in ctDNAs are potential targets for cancer detection, diagnosis, prognosis, and guidance for treatment of cancer, there are still barriers in the accurate detection of specific cell-free nucleic acids.[89] Under different conditions, such as variabilities in methods of detection, number, and types of targeted molecular alterations, tumor types, and stages the sensitivity of clinical detection has enormous challenges.[90] As cfDNA is highly fragmented DNA and the total amount of ctDNA is as low as 0.01% of the total cfDNA, the detection of such extremely low concentration ctDNA is difficult, particularly at the early stages of tumor development.[56] In addition to these challenges, the requirement of prior knowledge about particular mutations in ctDNA is another bottleneck of ctDNA based assays.[91]

For the clinical implementation of ctDNA based diagnosis of HCC, the test should have high sensitivity and specificity. However, the specificity of the test is challenging because most of the driver's genetic modifications are not liver-cancer specific. For instance mutation of TP53 are distributed in all coding exons of the TP53 gene which encodes the DNA-binding domain of the protein but, about 30% of the mutations fall within 6 "hotspot" residues (residues R175, G245, R248, R249, R273, and R282) which are frequent in almost all types of cancer.[92] 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 are used.[42]

Since clonal heterogeneity is a general phenomenon for tumor cells, it adds a level of complexity to our understanding of the biology of tumor development and poses challenges for the development of successful targeted therapy.[93] In HCC, different genetic and epigenetic alterations in individual tumor cells, together with selection pressure, may cause populations of tumor cells to undergo a molecularly heterogeneous transformation, even with the seemingly identical histopathological traits.[94,95] This evolution can start at different time points during the initiation and progression of tumors leading to the spatial and temporal heterogeneity of HCC. As a result, a single-site biopsy is certain to miss clinically important mutations from a heterogeneous HCC tumor.[37,74]

8. Conclusion

Hepatocellular carcinoma is the primary malignancy of the liver that occurs predominantly in chronic liver disease. It is the fifth most common cancer in men and the seventh in women. It has a high mortality rate due to a lack of therapeutic options and difficulty in early detection and prognosis. Currently, HCC diagnosis is made by radiologic imaging techniques and histology. However, small HCC cannot be proper diagnosed by such methods. Because radiologic imaging cannot present typical vascular patterns and histology requires repetitive biopsies for pathological interpretation. But in recent years, ctDNA, a liquid biopsy biomarker, is believed to be easily detected in the plasma of cancer patients. Since HCC is a disease caused by genetic and epigenetic DNA modifications like other cancers, identification of DNA modifications associated with HCC development permits an unambiguous diagnosis of HCC cases. The identification of such candidate DNA markers will be useful for either diagnosis or surveillance of HCC when they can be detected in the peripherals without liver biopsy which can be achieved by analyzing ctDNA from body fluids. Hence, analysis of tumor-specific genetic modifications, such as DNA methylation, microsatellite alterations, point mutations, and chromosomal rearrangements in ctDNA from body fluid is a promising finding which can be used for HCC diagnosis in the future. It can also be used for screening and detection of pre-symptomatic cancer at its

early stage. Despite the great potential of this technique, it has not been approved for routine clinical use.

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10. Conflict of Interest

The authors declare they have no conflict of interest.

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