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Clinical and Genomic Strategies for Detecting Hepatocellular Carcinoma in Early Stages: A Systematic Review

Esraa M. Hashem[1], Mai S. Mabrouk[1,*], Ayman M. Eldeib[2]

[1]Biomedical Engineering Department, Misr University for Science and Technology (MUST University), Egypt
[2]Systems and Biomedical Engineering, Faculty of Engineering, Cairo University, Giza, Egypt

Abstract Hepatocellular carcinoma is one of the commonest deadly tumors, and it is usually diagnose at a late stage, when effective treatment is very difficult. Making its discovery before the development of later stage disease is challenging. Hepatocellular carcinoma represents one of the prevalent type's cancers with the highest proportion in developing countries. There are many clinical and pathological staging systems for detecting Hepatocellular carcinoma, but none of them includes biological parameters as predictors for prognosis. The various clinical presentations commonly relate to the extent of hepatic reserve at time of diagnosis and so far, there are no studies that conclusively prove that screening hepatocellular carcinoma will reduce the death rate. The development of genome-wide analysis methods has opened the possibility of identifying multiple changes simultaneously in genetic or epigenetic alterations as well as in gene expression affecting the genome of cancer cells. The main issue raised by this work is to determine which alternatives can construed as reliable biomarkers for providing information about the carcinogenesis process rather using screening methods for detect hepatocellular carcinoma. This systematic review opens the door to Bioinformatics to discover novel cancer biomarkers that will help for early diagnosis of hepatocellular carcinoma. Identifying non-invasive and cost-effective biomarkers for early detection and personalized treatment of HCC will be one of the most promising fields of biomarker research. Find an effective, reliable tool for early diagnosis of HCC to increase the number of patients who are appropriate for therapeutic treatment will play a pivotal role in improving HCC patients' prognosis. The traditional approaches to identify genomic alternations suffered from several inherent disadvantages, Next generation DNA sequence promises to revolutionize cancer research, diagnosis and therapy.

Keywords Hepatocellular carcinoma, Biomarker, Screening methods, Bioinformatics, Next generation DNA sequence

1. Introduction

Hepatic cancer or liver cancer is a cancer that begins in the cells of the liver. Liver cancer consists of malignant hepatic tumors in or on the liver. Hepatocellular carcinoma (HCC) is the most common form of liver cancer, which begins in the main type of liver cell (hepatocyte). HCC classified as the fourth most common cancer in the world and responsible for about a quarter of a million deaths annually [1]. The survival of patients is extremely poor by the time they present with symptoms associated with tumor [2]. The understanding of the natural history of HCC hampered by the variability of the tumors, which is affected by the co-occurrence of many factors in the same patient as well as by the presence of multiple distinct cell lines in the liver that may develop into liver cell cancer [3].

* Corresponding author:
msm_eng@yahoo.com (Mai S. Mabrouk)
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The displaying of HCC has evolved significantly over the past few decades. While, in the past, HCC generally presented at an aggressive stage with right upper quadrant pain, weight loss, and signs of decompensate liver disease. Now HCC recognized at a much earlier stage because of the routine screening of patients with known cirrhosis, using serum alpha-fetoprotein and cross-sectional imaging studies measurements that will discuss later. Cancer is duo to genetic alternations accumulate in the cell. There is need to identify genes whose alternations accumulate during tumor progression to understand the molecular mechanisms of metastasis. Most cancer proceeds through accumulation of mutations in genes that cause death and this applied for HCC as well.

Etiology of hepatocellular carcinoma:

In Egypt, HCC is one of the most deadly cancer types. It is the second most common malignancy in males and the fifth in females (www. ncbi. edu.eg). Fig.1 shows the estimated percentage of liver cancer in Arab world. It shows that: in Egypt, liver cancer has higher rate prevalent cancer type especially in male.

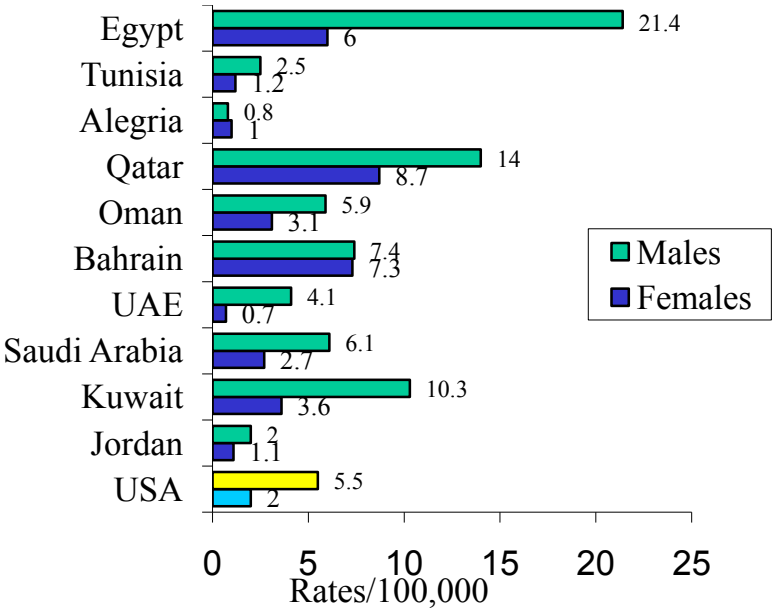

Figure 1. Magnitude of Liver Cancer in Arab world

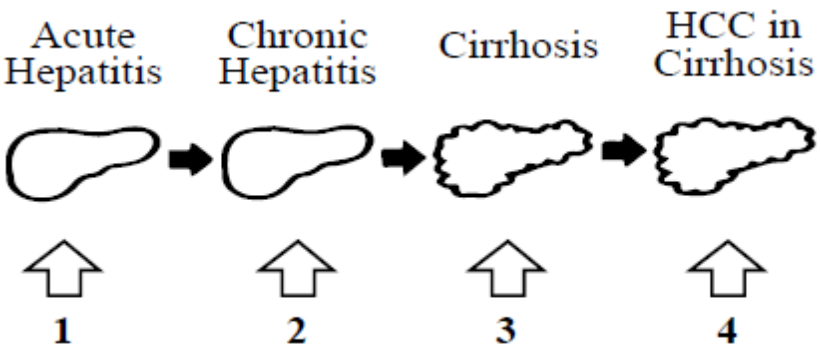

Figure 2. The stages of develop HCC

The main reasons for HCC development are Hepatitis B and C viral infection, aflatoxin B1 and alcohol, which are the most common underlying cause of chronic liver disease, which leading to liver cirrhosis and chronic hepatitis [4]. Liver cirrhosis is the most important predisposing factor for HCC as shown in Fig. 2 the genome of human HBV does not contain oncogenes and it is probably making its effect on hepatocarcinogensis of transactivation or trans-repression of cellular genes of factors by HBV-related gene products.

HCV is a single positive-stranded RNA virus. These risk factors can induce damage in DNA sequences and mutations, such as p53 mutation induced by aflatoxin and DNA damage induced by the intrusion of the HBV genome [5]. HCV infection affects 3:4 million people yearly and about 170 million people are chronically infected with virus C [6]. The tumor size when HCC is first detected, does not predict the course of the disease in all cases. In fact, the median time of doubling volume for a small HCC may range from 1 to 20 months [7]. Detection and characterization of tumor vascularity are important in the differential ways: diagnosis, the choice of treatment method, and assessment of the therapeutic response for HCC.

One of the main issues about HCC is whether screening for HCC can improve patient survival or not. Early detection is important for patients who are at high risk of having HCC. For HCC, many studies limited by lead-time bias. To date, no substantial evidence has accumulated that improves survival benefit with surveillance of high-risk patients. There are diagnostic tools used commonly try to improve patient survival: serum tumor marker alfa-fetoprotein (AFP), radiographic imaging, liver biopsy and biomarkers.

The main purpose of this systematic review is to explain common methods (invasive and noninvasive) used clinically to detect HCC and their limitations. It provides the reader with an appreciation of the contributions of molecular cytogenetic, that making the assessment of biomarkers in which chromosomal rearrangements are affecting the molecular biology and clinical response of tumors. It opens the door to Next Generation Sequence (NGS) technologies. It promises a global view on oncogenomics by facilitating

integrative and efficient detection of epigenetic and genetic alterations in cancer at a single-base resolution that can help in early detection of HCC.

Cancer Biomarkers:

The accessibility of the complete human genome has opened the door to understand many diseases. As recent technological progress in proteomics and functional genomics have fuelled interest in recognizing the biomarkers of complex diseases such as liver cancer, which usually caused death. Bioinformatics holds a great interest in the scientific community because it is less expense than traditional laboratory experiments and its ability to move scientific research forward more rapidly [8]. A biomarker might be either a molecule secreted by a tumor a specific response of the body to the presence of cancer. These markers not only help in prediction of prognosis of cancer but may also assist in deciding appropriate method of therapy and may represent novel potential targets for therapeutic interventions. In the recent years, knowledge about cancer biomarkers has increased enormously providing great opportunities for improving the management of cancer patients by enhancing the efficiency of detection and efficacy of treatment [9].

Most HCC patients currently identified in clinics are usually at an advanced stage of the disease with a very poor prognostic outcome. For this purpose, early detection using a tumor marker is essential to reduce the mortality of this disease. The most widely used cancer biomarkers for liver cancer or HCC known so far are: AFP, AFLP (*Lens culinaris* – a derivative for AFLP), des- carboxyprothrombin (DCP), Golgi protein 73 (GP73), and Glypican-3 (GPC3) [10-11].

Recent developments in gene expression microarray, proteomics and genomics permit thousands of genes and proteins to screen simultaneously. Moreover, new biomarkers expected to be arising in the next years for the screening of many cancers including HCC.

Human cancers are mainly genetic diseases caused by genome alterations: DNA sequence changes, chromosomal rearrangements, copy number aberrations, and epigenetic modifications (DNA methylation). Together, they drive the development and progression of malignant transformation [12].

The function of Bioinformatics in the prediction of new cancer biomarkers as illustrated in Fig. 3 will also increase and become more and more important in the early disease diagnose. Many Bioinformatics methods are analyzing data for two main targets: to discover molecular targets for designing therapeutic intervention and to identify reliable cancer biomarkers for early diagnosis of cancer. The analyses in Bioinformatics focus on three types of large datasets available in molecular biology: ***genome sequences, molecular structures, and gene expression data.***

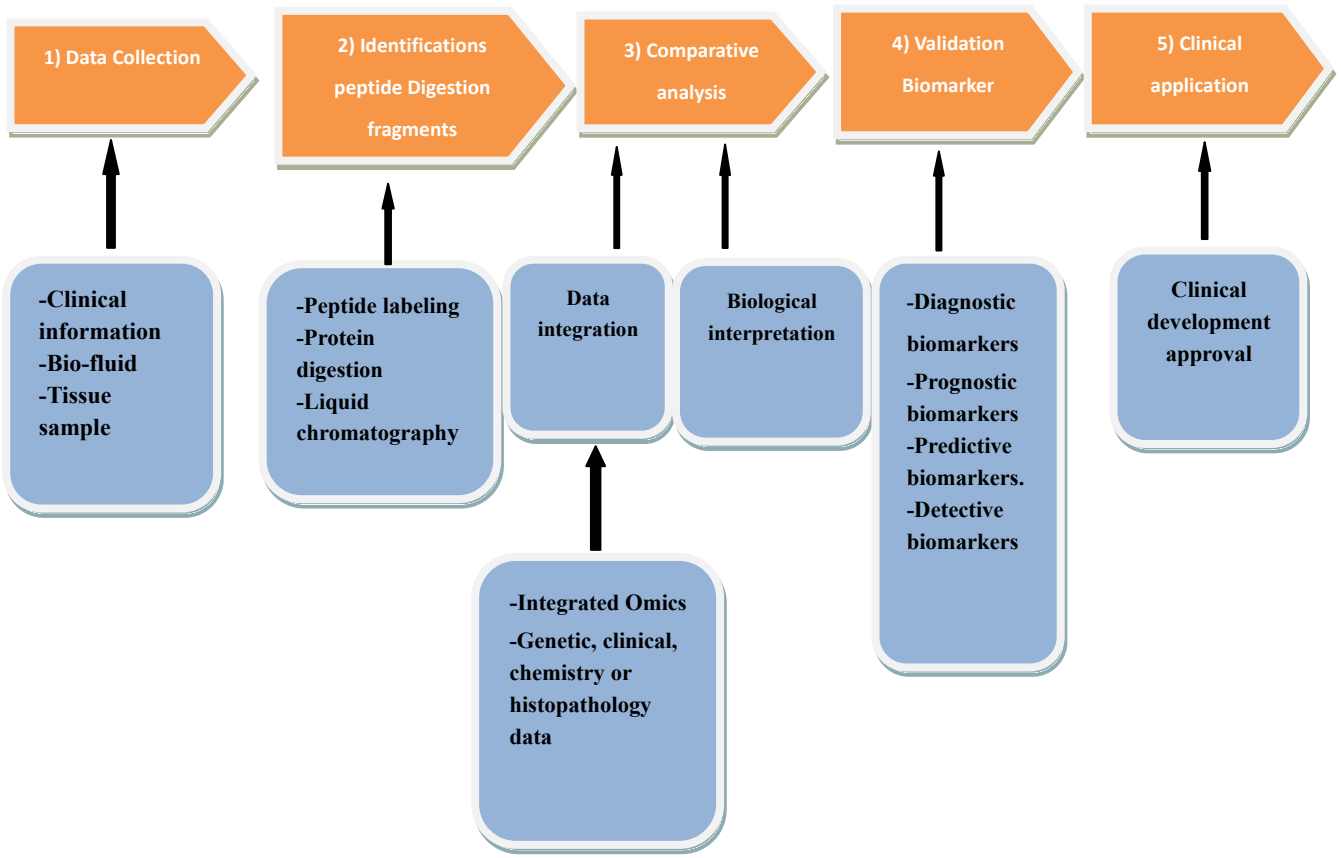

Figure 3. Biomarker discovery in a cross-disciplinary domain

There are clinical methods to detect HCC as invasive and non-invasive methods that considered as old techniques for screening liver cancer. Long-term survival of HCC patients is poor. This is partly due to recurrence of HCC. Usually HCC diagnosed at late stage and for this reason; its challenge is to discover HCC in early stage. According to current studies, the majority of HCC patients get the disease from the accumulation of genetic abnormalities that have methodologists for HCC biomarker discovery [8], and this will be discuss in later section.

2. Clinical Methods to Detect HCC

Invasive Method

Liver biopsy: considered as an invasive approach used for histological scoring and still used as reference test for fibrosis staging. A liver biopsy procedure is to hold a small piece of liver tissue for examination with a microscope for signs of damage or disease. There are three types of liver biopsy that are: Transvenous, Laparoscopic and percutaneous biopsy. Percutaneous biopsy considered as the most common type of liver biopsy as shown in Fig 4 biopsy is a cornerstone in the evaluation and management of patients with liver disease and it has considered a key element of the clinician's diagnostic armamentarium [13]. Unfortunately, procedure of liver biopsy is invasive, expensive with serious side effects leading to death through possible (internal bleeding) and not suitable for all patients.

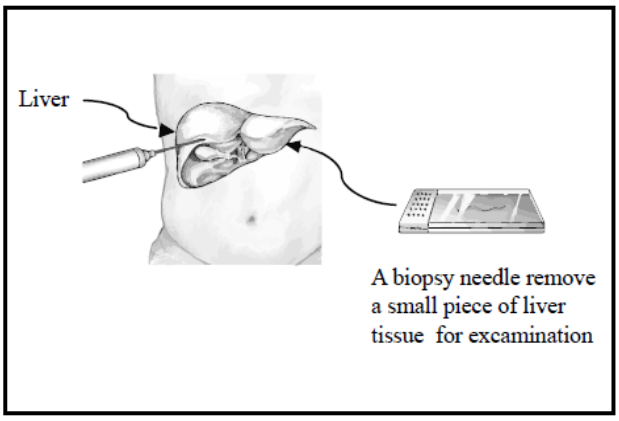

Figure 4. Percutenios liver biopsy procedures

Non- Invasive Methods and diagnosis performance

As patients suffering from chronic hepatitis or cirrhosis may develop HCC only after many years, emphasis has placed on the early detection of HCC when it is small, asymptomatic and potentially curable, through the screening of patients at high risk [14]. Knowledge of the biology of tumor progression allows us to identify certain tests that are useful for early detection or screening of HCC. There are three methodologies have been employed in HCC surveillance for high-risk patients: specifically serum AFP concentration, diagnostic imaging (screening method) and tumor marker determination.

1-Alfa-Fetoprotein

Since 1968, AFP has been use as a serum marker in the detection of human in HCC [15]. Serum AFP is a protein produced mainly through fetal liver. Serum AFP is the first serologic assay for detection and clinical follow up of patients with HCC, and it has been the standard tumor biomarker for HCC for many years. The reported sensitivity and specificity values necessarily vary depending on the cutoff level chosen for the diagnosis of HCC. A radical disadvantage of AFP as a tumor marker is the fact that serum AFP levels can increase in patent that have active hepatitis, but not HCC. Serum AFP is used in the diagnosis and screening of liver cancer because AFP is above the normal range in 60.0% to 70.0% of primary liver cancer cases. One of the problems with AFP is in defining the cutoff level for the diagnosis of HCC, defining >500 ng/ml as a diagnostic level for HCC is unsatisfactory because many patients with early HCC have the AFP levels well under 500 ng/ml [16].

Furthermore, patients with chronic hepatitis B or C with reactivation sometimes have AFP levels >500 ng/ml. For that is reason and other, AFP determination has been frequently rejected as a screening test for HCC. Total AFP have three different types glycoforms, AFP-L1, AFP-L2, and AFP-L3-based on their binding capability to lectin -Lens culinaris -agglutinin (LCA). High percentage of AFP-L3 found to be associated with poor differentiation and biologically malignant characteristics, worse liver function, and larger tumor mass [17].

2-Ultrasonography and alpha-fetoprotein

The ultrasonography is Ultrasound (US) based on diagnostic imaging technique. Sonography plays an important role in the evaluation and follow-up of patients with known liver cirrhosis, Focal fatty infiltration, HCC, regenerating nodules (adenomatous hyperplasic nodule), or other focal lesions may occur in a cirrhotic liver [18]. The most common tests used for screening are ultrasonography of the liver and serum alpha-fetoprotein (AFP). Ultrasonography can detect lesions as small as 1 cm inside the liver, however; it is often difficult to distinguish HCC from other conditions. Sonography has low sensitivity but high specificity in revealing HCC and dysplastic nodules in patients with a cirrhotic liver requiring liver transplantation [19].

The sensitivities of both ultrasonography and AFP to detect early HCC are not satisfactory. Despite their limitations, studies using one or both screening methods show that screening can detect HCC at an earlier stage, increase the opportunity of receiving curative treatment, and improve survival [20]. The sonography have been replaced in diagnosis by Computed Tomography (CT) scan and Magnetic resonance imaging (MRI) as a diagnostic instrument of choice as a result of low sensitivity and positive predictive value with coexisting cirrhosis [21].

3-Computed Tomography

The computed tomography is a diagnostic medical test like traditional x-rays. CT generates multiple images of the inside of the body. A CT scan uses a combination of x- rays and computer technology to create those images. CT is consider as attractive imaging modality for HCC screening because it can detect lesions in the cirrhotic liver [22]. Fig.5 shows CT scan in liver of patient with HCC [23]. A study on hepatitis C cirrhotic demonstrated CT scan imaging to have a higher sensitivity for detecting HCC than either ultra sound or AFP when used alone (88% vs. 59% and 62%, respectively) [24]. Brancatelli et al .describe hepatic lesions that can simulate HCC on CT imaging including regenerating nodules, focal fat, hemangiomas and dysplastic nodules [21]. CT's limitation include the need for a high radiation dose, less sensitive for the detection of small HCC and for dysplastic nodules, which appear, is dense to the liver parenchyma due to their predominant blood supply from the portal vein [25].

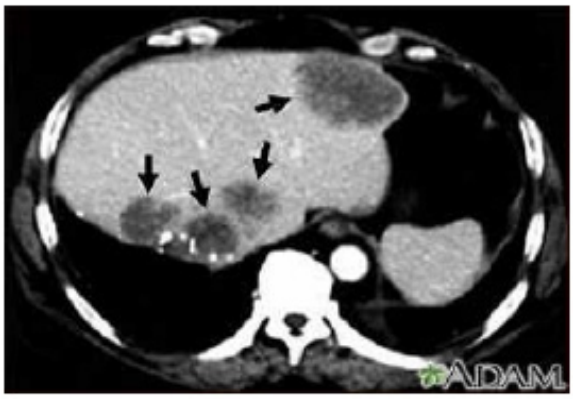

Figure 5. CT scan of the upper abdomen showing cancer that has spread in the liver of a patient with carcinoma of the large bowel

4-Magnetic Resonance Imaging

Magnetic resonance image scans use radio waves and strong magnets rather than x-rays. The energy that comes from the radio waves is absorbed and released in a pattern formed by the type of body tissue and by some diseases. A computer translates the pattern into a highly detailed image of parts of the body [26]. Fig.6 shows the enhancement of the tumor during the arterial phase for HCC patient [27]. MRI is better than CT in providing structural information on unenhanced images. MRI has led to significantly better detection of liver lesions following improvements in technology and techniques [28]. MRI has become the diagnostic imaging mode preferred for HCC at many institutions around the world [21]. Many studies have shown the superiority of MRI in both lesion detection and characterization for focal hepatic lesions when compared to CT [29-30].

The sensitivity and specificity are similar to those of multiphasic CT scan imaging, MRI sensitivity is lowest when evaluating tumors less than ‹2 cm in diameter [21]. The main defects of MRI include that it is a long procedure time, very expensive, and the need for the patient to hold his breath for longer periods [26].

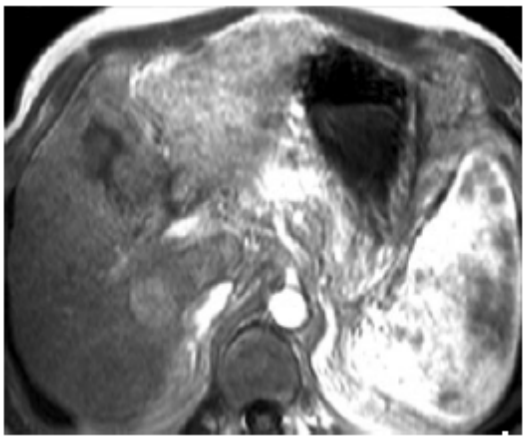

Figure 6. Contrast MRI shows transient enhancement of the tumor during the arterial phase for HCC patient

5-Ultrasound elastography (Fibroscan)

Fibroscan is a new imaging technique that allows a noninvasive estimation and imaging of tissue elasticity distribution during biological tissues using conventional real-time ultrasound equipment with modified software [31]. It is an ultrasound device that measuring shear wave velocity. A 50-MHz wave passed into the liver from a small transducer on the end of an ultrasound probe as shown in Fig. 7 this technology measures the velocity of the sound wave passing through the liver and then turn out that measurement into a liver stiffness measurement. The whole process often referred to as liver ultrasonographic elastography [32].

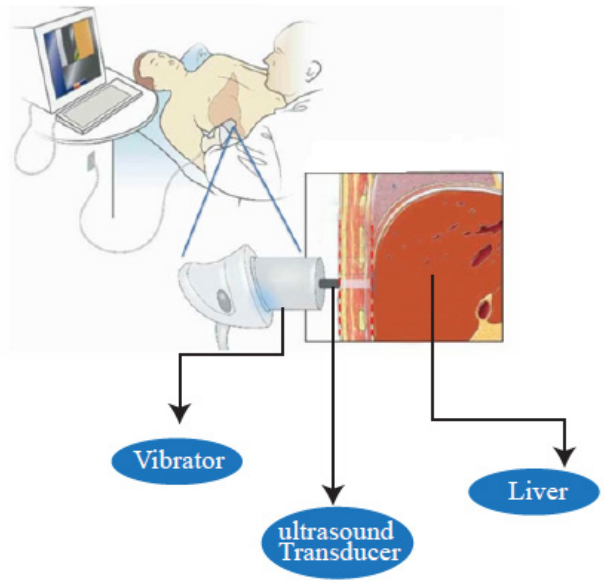

Figure 7. The ultrasound transducer is positioned in an intercostal space near the right lobe of the liver

Liana. G, et al. estimate real-time elastography as a noninvasive tool for the diagnosis of small (1-3 cm) HCC nodules in cirrhotic patients [33]. Furthermore, they aimed to

identify predictors of HCC diagnosis in nodules less than 3cm in diameter detected at US examination in cirrhosis. In conclusion, elastography imaging of the small liver nodules in cirrhosis can used as a non-invasive tool for accurate diagnosis as well as selection of patients for curative therapy, but not for screening. Its utility in patients with non-vascularized nodules can help to eliminate the false negative diagnosis of enhanced contrast imaging of the liver and to improve their outcome.

The Fibroscan has not been associated with any side effects and it is much less expensive than liver biopsy. The results of the test taken immediately. Therefore, clinicians can use these results to make decisions during patient's visit. However, there are some limitations of using ultrasound elastography, which are: it may over estimate damage in acute HCV and it is not recommended for patients with ascites, and/or morbid obesity. Table.1 summarizes all screening techniques used to detect HCC with their limitations. Unlike other solid malignancies, cirrhosis and the coexistence of inflammation make the early diagnosis and prognostic assessment of HCC much more difficult. There is no definite evidence that screening in HCC improves survival [34]. This complication highlights the urgently need to identify valuable biomarkers for the diagnosis and treatment of HCC to improve survival.

Table 1. Summarized imaging techniques with their limitations

Screening method	Technique	Limitations
AFP	Serum marker	can increase in patent that have active hepatitis
Ultrasonography and AFP		Low sensitivity
CT	Identify micro vascular Permeability changes	Cannot performed in renal failure and contrast agent allergic patient
MRI	Observe change in hepatic Parenchyma	High cost
Ultrasound elestography	Used conventional real-time ultrasound equipment with modified software	

3. Pathological Methodologists for HCC Biomarker Detection

DNA sequencing data is complex genome with a large number of mutations. The biological interaction and clinical significance of the overall aberrations are largely unknown. The general practice among many physicians has been to screen HCC using ultrasound and serum a-fetoprotein (AFP) its sensitivity and specificity are poor [35]. However, many patients still exist with either large HCC (>5 cm) or multifocal HCC (more than three lesions) or HCC that has invaded the portal vein or other critical structures. Genetic abnormality, epigenetic, proteomic and imaging biomarkers can used for cancer diagnosis, epidemiology, and prognosis. Ideally, such **biomarkers** can assayed in noninvasively collected bio-fluids like blood or serum [36].

Genomic Instability of HCC: Genomics instability has become a prevailing method for understanding cancer biology and providing cancer classification according to biological differences. The complex nature of cancers that initiated by activate many different oncogenes or inactivate different tumor suppressor genes and progress by additional epigenetic or genetic alterations. Traditional gene-by-gene approaches are likely to deliver only a limited understanding of the biological and pathological characteristics of cancer cells.

However, the latest development of technologies for gene expression profiling, genome copy number analysis, and whole-genome sequencing, enable the comprehensive characterization of entire cancer genomes. This information will help to improve our understanding of the epigenetic and genetic alterations driving the development of HCC, as well as ultimately to provide information that help in treatment of patients [37]. The genomic instability use epigenetic alternation (DNA methylation), gene expression, and chromosome aberration for estimate genetic and epigenetic alternations.

Epigenetic Alternation

Epigenetic modifications describe changes in DNA/ chromatin that do not involve changes in DNA sequence. Epigenetic alternations affect the gene transcription and chromatin structure without change the sequence of the genome [38]. DNA methylation is the main aspect of epigenetic alternation. Methylation normally occurs at the fifth carbon atom in the nucleotide base cytosine (5-methylcytosine) [38]. DNA methylation is using in silencing gene and chromatin remodeling. Gao *et al*. applied methylated CpG island amplification microarrays to investigate DNA methylation in cancerous and precancerous tissues from HCC [39]. They found aberrant DNA methylation of several important genes, such as *DUSP2* and *BMP6*, which are known to be associated with tumor genesis.

DNA methylation status significantly different between HBV- and HCV-positive patients with HCCs in both cancerous tissue and noncancerous tissue, suggesting that the HBV-related carcinogenetic pathway may result in distinct DNA methylation profile [40]. Increasing evidences indicated that epigenetic alterations are developing as an important mechanism in cancer initiation and progression [41].

Many studies indicated that hyper-methylation might be essential mechanism involved in the inactivation of some tumor suppressor's genes in HCC. Fig. 8 lists gene, which have been report frequently to involve DNA methylation in HCC [42].

Microarray and Gene Expression profiling

Microarray is a technique used in molecular biology to query the expression of thousands of genes simultaneously.

A genomic technique that based on fact that only a part of the genes encoded in the genome of a cell are expressed by being cloned into messenger RNA (mRNA). The complement of mRNAs in a cell largely dictates its complement of proteins. Thus, gene expression is a major determinant of the biology of both normal and malignant cells.

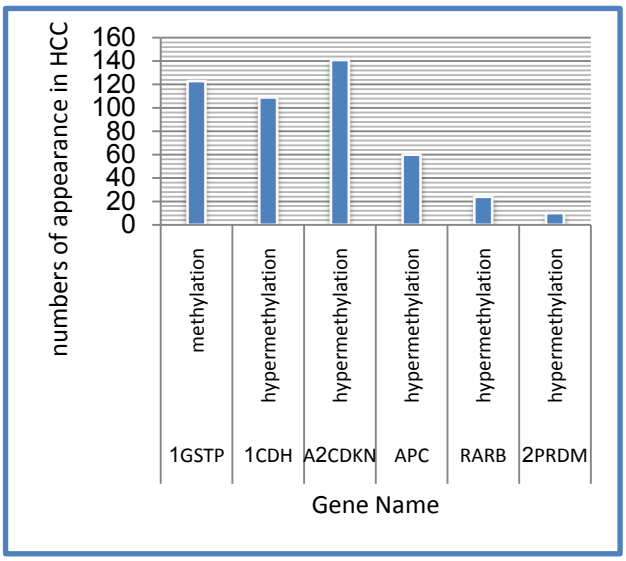

Figure 8. List of abnormality aberrant gens reported in HCC

The use of gene expression profiling is particular importance in cancer. This is because the initiation and malignant progression of cancers result from the combining and accumulation effects of many changes in the sequence or expression level of cancer commencement genes [43]. Hsp70 considered as a sensitive marker for the differential diagnosis of early HCC from a precancerous lesion or a non-cancerous liver [44].

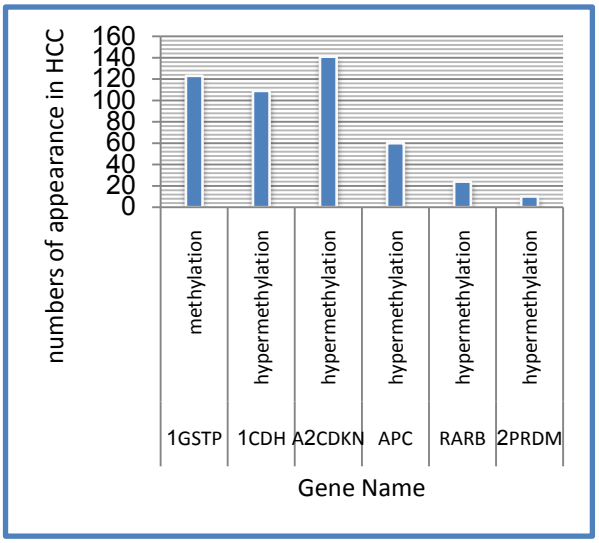

Figure 9. Genes appeared in some HCC microarray studies

There are some techniques available to monitor gene expression on a genomic scale such as, suppression subtractive hybridization (SSH) [45], and differential display (DD) to analyze hepatocarcinogenesis [46, 47]. Caillot et al. used microarray technique and found a significant association of SERPINF2, ITIH1, and TTR genes expression and their related proteins with all fibrosis stages of liver [48]. Fig.9 shows list of genes appeared in HCC microarray study [42].

Chromosomal aberrations

Chromosomal aberrations are the most common type of genetic alteration. Copy number alternation (CNA) corresponds to comparatively large regions of the genome that have been delete or duplicated on certain chromosomes. In addition, it is represent by loss or gain of a chromosomal segment during cell division and leads to an increase in aneuploidy, which in turn cause more mutations and enhance tumor progression leading to aggressive tumors. HCC demonstrates a high incidence of chromosome aberration. Identifying such aberrations can help to locate diagnostically important regions in the genome, which harbor differentially expressed genes. These aberrations include large chromosomal gains, losses, amplifications and deletions. Genomic instability can also lead to over expression or activation of oncogenes and the silencing of tumor suppressors.

During cell replication, many types of errors can occur that led either to the insert of extra copy or deletion of part of a DNA sequence in the genome. If the errors remain unchecked, these errors can silence important genes or amplify their expression, in both cases leading to an improperly functioning cell. If they involve amplification of proto-oncogenes that regulate cell division or deletion of tumor suppressor genes that prevent undesirable cell division or caused programmed cell death, these errors are factors contributing in the initiation stage of carcinogenesis.

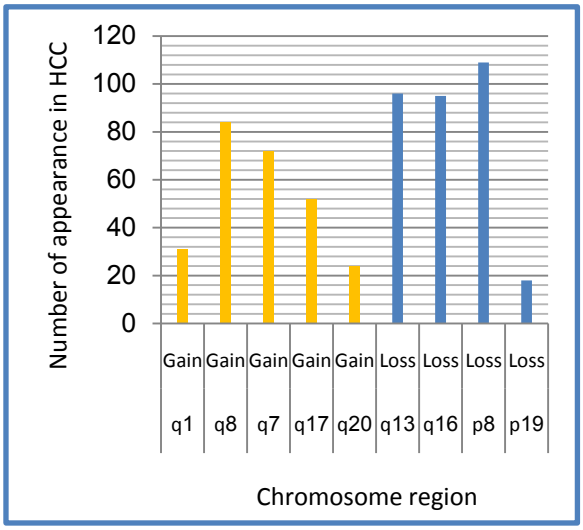

Figure 10. List of chromosome aberration detected of some studies in HCC

Accumulation of particular certain of these genetic errors can cause a group of cells to cross the threshold to cancer, at the point the cells' increased genetic instability and high replication rate will lead to even more errors, which can lead to progression or metastasis [49]. This genetic change can

observed as early as in the pre-neoplastic lesions of cirrhotic liver, and it believed to be the initiating events in hepatocarcinogenesis. Genomic DNA copy number gains, amplifications or loss are shows to play important roles during development of HCC.

Cytogenetic abnormality is biomarkers that can be detected by: fluorescence in situ hybridization (FISH), Comparative Genomic Hybridization (CGH) and Array Comparative Genomic Hybridization (a-CGH). Fig. 10 lists the aberration region frequently detected in HCC copy number variation showing the main types of aberrations loss and gain.

DNA Copy Number Alternations (*CNA*):

Copy number alterations are genetic disorders that closely correlate with many human diseases [50].CNAs can be discover by cytogenetic techniques. A number of methods are currently available to detect CNA: fluorescent in situ hybridization, comparative genomic hybridization, array -CGH, and by virtual karyotyping with single nucleotide polymorphs (SNP) arrays. Lately, DNA sequencing technologies have enabled the identification of CNVs by next-generation sequencing [51-52].

DNA copy number alternations can be definitely as stretches of DNA larger than 1 kB that display copy number differences in the normal population [53]. Identification of cancer specific CNAs will not only provide new insight into understanding the molecular basis of tumorigenesis but also assist the discovery of new cancer genes. CNAs change the level of gene expression, which modifies normal growth control and survival pathways. CNA can detect through calculate the intensity log2 ratio of chromosomes [54].

Subharup. G et al. develop a statistical framework for detecting copy number gains and losses, identifying localized deletions and amplifications, and partitioning tumor DNA into regions of relatively stable copy number rely on the hidden Markov model (HMM) to calculate the dependence between neighboring clones. They found that the sensitivity of the algorithm to individual probes often allows them to find candidate genes that missed by other algorithms [55].

A. Fluorescence in situ hybridization (FISH)

Improvements in cloning technologies and antibody coupling in the late 1970s and early 1980s led to the introduction of FISH as early as 1977 [56]. By the late 1980s, FISH was being used to detect specific chromosomal regions and loci [57] using recombinant libraries, enabling the chromosomal mapping of many genes [58-59] within the human genome. FISH uses fluorescent DNA probes to target specific chromosomal positions within the nucleus, resulting in colored signals that can be detect using a fluorescent microscope.

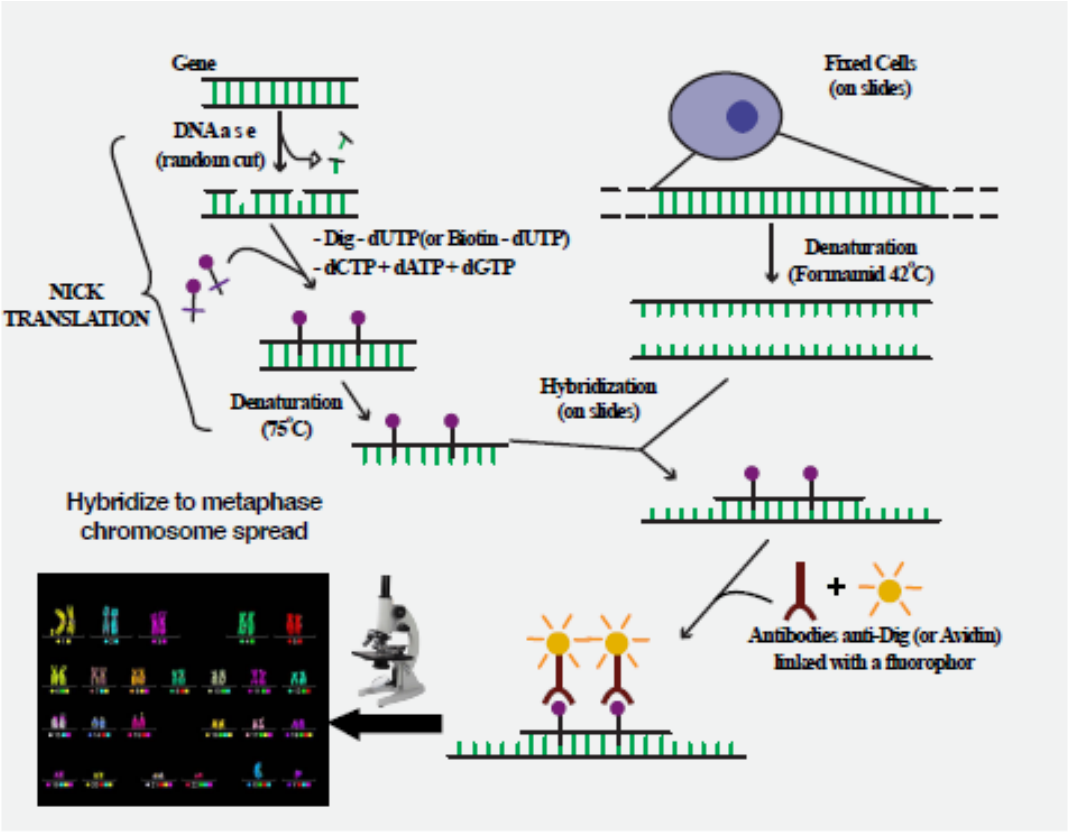

Figure 11. Scheme of the principle of the FISH Experiment to localize a gene in the nucleus

The fluorescence in situ hybridization has the ability microscopically to visualize the location of individual sequences on metaphase chromosomes. This technique can also use to detect chromosome abnormalities or determine the chromosome location of specific sequence and to identify chromosomes. Fluorescence in situ hybridization can be scaled up to analyze hundreds of cells and thereby increase the sensitivity of analysis and the detection of clone chromosomal abnormalities in far greater numbers of cells than traditional chromosome banding techniques and metaphase analysis. Fig. 11 shows all the preparation process for FISH method to detect copy number alternation.

Ludwig. W. et al. performed FISH on interphase nuclei of cytological specimens enabling the correct detection of copies of chromosome in tumor cells of 18 moderately (G2), well (G1), or poorly (G3) differentiated hepatocellular carcinomas. They found that there was a close correlation between the morphological dedifferentiation and increase in copy numbers as well as difference of FISH signals for chromosomes 1 and 8 [60]. FISH may not have high enough resolution to identify the exact chromosomal region involved in abnormalities [61].

B. Comparative genomic hybridization (CGH)

CGH is a fluorescent molecular cytogenetic technology for determining copy number gains, losses and amplifications between two samples of DNA by competitively hybridizing differentially labeled DNA from these samples to normal metaphase chromosomes [54].

Tumor DNA is labeled with a green fluorochrome, which is subsequently mixed (1:1) with red labeled normal DNA and hybridized to normal human metaphase arrangements. The green and red labeled DNA fragments compete for hybridization to their position of origin on the chromosomes. The green to red fluorescence ratio measured along the chromosomal axis represents the relative DNA copy number in test versus the reference DNA of genetic material in the tumor at that specific locus [62].as shown in Fig.12.

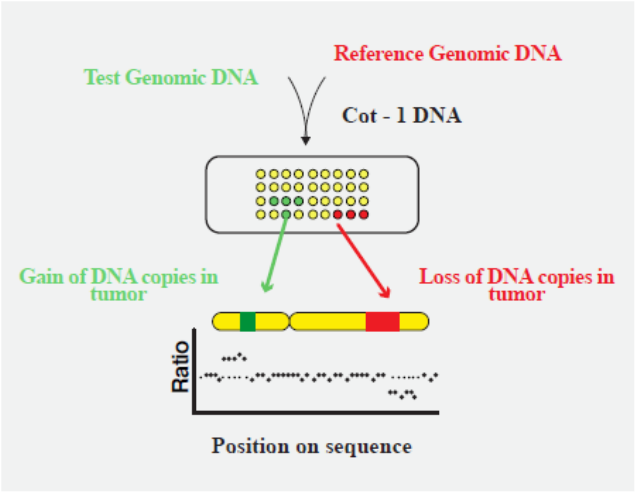

Figure 12. Schematic representation of Comparative Genomic Hybridization

Kusano N. et al, performed CGH analysis in 41 HCCs to examine whether the analysis of cytogenetic aberrations allows us to evaluate biologic behavior of HCC. They detected loss at 13q, amplification at 11q13 and loss at 8p chromosomes. This search denotes that cytogenetic information provided by comparative genomic hybridization is useful for estimating prognosis of patients with HCC [63].

There are limitations of using CGH: It cannot detect structural chromosomal aberrations without copy number changes, such as balanced chromosomal translocations, ring chromosomes or inversions. It does not give information in the context of tissue architecture. The resolution of conventional CGH restricted by the length of the metaphase chromosomes, which is approximately 10 mega bases and could have hundreds of genes. Array comparative genomic hybridization based on the same principles as CGH, except that the targets are mapped genomic clones instead of whole chromosomes.

C. Array-Comparative Genomic Hybridization

Array comparative genomic hybridization is a prevalent technique used to identify chromosomal aberrations in human diseases, including cancer. The competitive hybridization strategy identified regions of genomic gain and loss, revealing regions harboring potential tumor suppressor genes (CGH regions of genomic deletion) and oncogenes (CGH regions of genomic gain/amplification) [64].

A-CGH is the most common method to investigate the number of copies of sequences of DNA. It was first described in Kallioniemi *et al*. [65], and then further described in Albertson and Pinkel [66] and Snijders *et al*. [67]. Recent array CGH studies of mantle cell lymphoma have detected a 50% higher number of chromosome aberrations than traditional CGH [68]. The a-CGH significantly improves the resolution (approximately 1 Mb) of the technique by replacing the hybridization target, the metaphase chromosome spread, with genomic segments spotted in an array format [69].

It provides numerous features including: easier standardization, higher resolution, and Array-CGH intensity ratios provide very useful information about genome-wide changes in copy number in DNA by calculating the intensity log ratio of chromosome [68]. A typical application of microarrays in cancer study is to classify patients with different disease status. On HCC, these studies include the classification of cancer vs. non-tumor samples, early stage vs. late stage, good prognosis vs. poor prognosis, high histological grade vs. low grade, and HCC patients with HBV vs. HCV infection, and others. Kim *et al.* identified 44 genes that can distinguish HBV-positive HCC from non-tumor liver tissues using cDNA microarray data analysis with a supervised machine learning method. [70].

Fig.13 shows the steps of a-CGH as: labeled test and reference genomic DNA by different colors, mixed, co-precipitated with unlabeled Cot-1 DNA to enable the blocking of repetitive sequences. There are enough amount

of Cot-1 DNA that is important to obtain adequate suppression of the repetitive sequences [71].

Application of a-CGH is widespread in molecular analysis of cancer and herald great promise as a technique to identify clinically related diagnostic biomarkers. There are many automated algorithms used to describe genomic profiles as increase the amounts of array-CGH data become available for instance, Cheng et al. discuss a regression-based test for altered copy numbers [72]. Jong et al. propose a break-point model to segment the clones [73]. Pollack et al. proposed a threshold method for identifying clones having an extreme value of emissions [74]. Lingjaerde et al. performed smoothing using the signs of neighboring data values and inspecting the width and magnitude of the segments to reveal regions of copy number change [75].

Esraa. M. H. et al. uses a CBS algorithm to detect the copy number alternation of HCC in order to improve HCC prognosis, and find useful biomarkers for monitoring of recurrence and early diagnosis [76]. The results show that CBS is effective in detecting global trends and this important feature for identifying biomarkers genes associated with cancer.

Mai S. M.et al. applied discrete stationary wavelet transform (DSWT) technique to a number of human chromosomes for analyzing array-CGH data to evaluate the prognosis of HCC patients in order to identify genome wide alternations in copy number of the genomic data [54]. The results show that DSWT is a powerful technology that provides more information about the development of HCC, which may provide better diagnosis and prognosis of HCC. The use of Discrete Stationary Wavelet Analysis modeling of a-CGH Data on HCC could open new frontiers in the identification of HCC biomarker genes [54]. Schizophrenia [77], cancer [78], array-CGH with next generation sequence (NGS) method to detect CNVs and found that NGS provides higher resolution of genomic regions having CNVs.

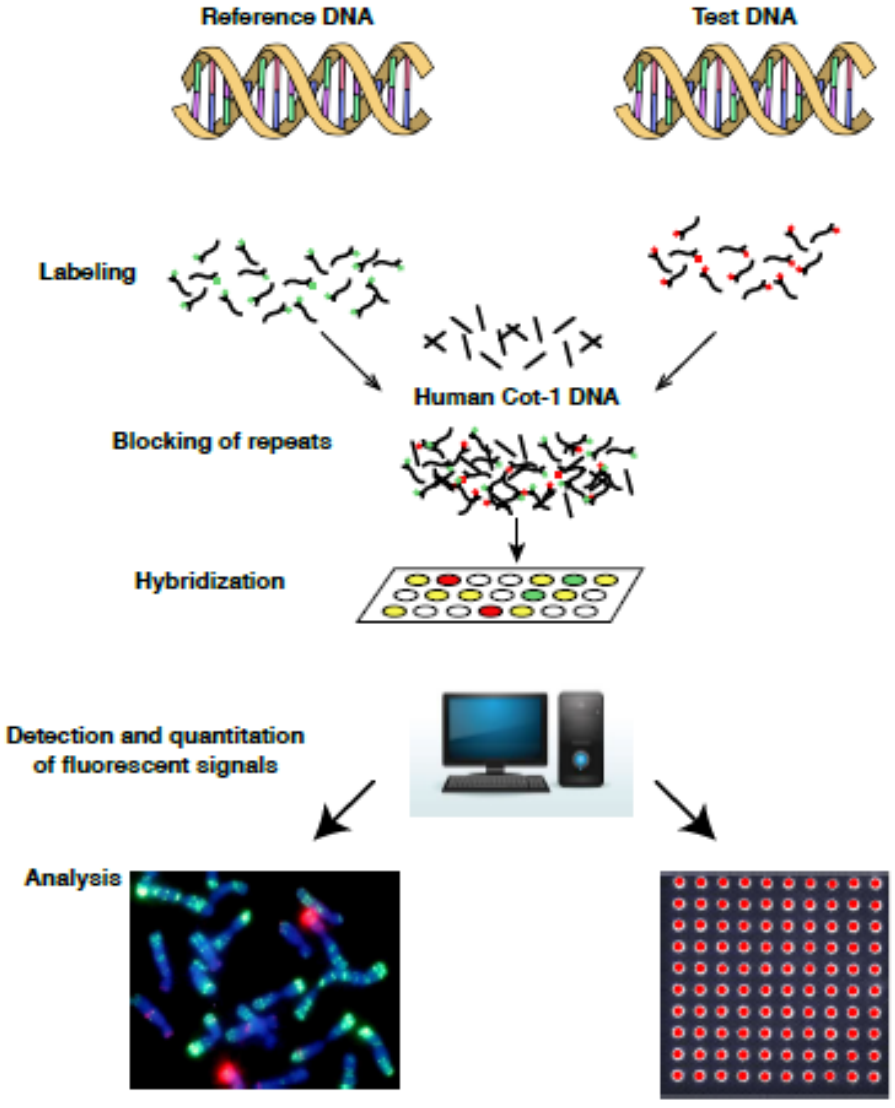

Figure 13. Arrays enable genome-wide analysis of DNA sequence copy number in a single experiment

D. Next generation sequence (NGS) approaches in liver cancer diagnosis

NGS depends largely on sophisticated bioinformatics analysis programs and faces significant data management and interpretation challenges for confirming whether a specific position in a single cancer-related gene is wild type, an SNP, or a somatic mutation [79]. This powerful technology used to determine the potential driver mutations in the development of liver cancer through Whole-Genome Sequencing (WGS) and Whole-exome Sequencing (WES).

Through the last few years, next-generation sequencing has included comprehensive characterization of copy number variations by generating millions of short reads in a single run and has evolved into a popular strategy for genotyping [80]. A great strength of next generation sequence approaches is that, they not only can detect base substitutions but also can simultaneously find CNAs, insertions, deletions, and translocations.

The number of studies where NGS technologies have been applied to investigate HCC are limited until now [81]. In 2011; the first primary liver cancer genome was sequenced and diagnosed with HCV positive HCC [82]. Massively parallel 50 base pairs paired end reads and lymphocytes obtained from the same patient, revealing 11,731 tumor-enriched somatic mutations. The resulting proof that NGS has proven powerful in the detection of viral infection in liver biopsies.

Different companies developed many different technologies such as: Illumina (Genome Analyzer II; San Diego, CA, USA), Life Technologies (Sequencing by Oligonucleotide Ligation and Detection or SOLiD), Roche Applied Science (454 Genome Sequencer FLX System; Indianapolis, IN, USA), Carlsbad, CA, USA), Ion Torrent Systems (now owned by Life Technologies, Ion Proton System), and Helicos BioSciences (HeliScope Single Molecule Sequencer; Cambridge, MA, USA) [83].

Zhengyan Kan. et al. report findings from a whole-genome sequencing study of 88 matched HCC tumor/normal pairs, 81 of which are HBV positive, pursuit to identify genetically altered genes and pathways implicated in HBV-associated HCC [84]. That study identifies also a number of prevalent and potentially actionable mutations, including activating mutations of (Janus kinase 1) and provides a path toward therapeutic intervention of the HCC disease.

Next generation sequence provided outstanding opportunities to investigate all sequences of entire cancer genomes to uncover the genetic changes that occur during cancer development, which can help to discover new biomarkers and to understand disease process for better diagnosis. Next generation sequencing technologies promise a global view on oncogenomics by facilitating integrative and efficient detection of epigenetic and genetic alterations in cancer at a single-base resolution that can help in early detection of HCC.

4. Discussion

HCC is the most common deadly cancers worldwide. It is responsible for a large percentage of cancer deaths worldwide. HCC is aggressive cancer, which frequently occurs in the setting of chronic liver disease and cirrhosis. The liver carried out several necessary functions, including detoxifying harmful substances in the body, cleaning blood and making vital nutrients. Cirrhosis is the end stage of chronic liver damage caused by chronic liver disease. Common causes of chronic liver disease are hepatitis C, hepatitis B infection. Identification of early HCC, which is potentially amenable to aggressive intervention and improved survival, are the reasons behind screening for HCC.

Liver biopsy considered as an invasive method for detect HCC. Unfortunately, procedure of liver biopsy can cause internal bleeding and not suitable for all patients. The diagnosis of HCC without pathologic confirmation can be achieved by assessing the serum AFP level combined with imaging techniques, including ultrasonography, computerized tomography and magnetic resonance imaging, [19, 23, 26]. However, improvement in early diagnosis is still require, because only 44% of the patients are diagnosed at an early disease stage, and only 30% of patients with HCC are candidates for potentially therapeutic treatments at the time of diagnosis [85]. Because of that, the application of new technology to improve our knowledge about the molecular pathogenesis of HCC, to identify biomarkers leading to an early diagnosis, and to define new curative targets, has considerable attention.

The large number of structural abnormalities present in cancer genomes is significantly attribute to genomic instability. Genomic DNA copy number alterations are associated with many complex diseases including liver cancer, as genetic alternations (amplifications and deletions) frequently contribute to tumor genesis, So there are a great need for sensitive, high-resolution techniques able to detect genomic instability by the time as this would afford critical insights into the mechanisms that underling genomic instability and the role of instability in tumorigenesis. The traditional approach to identify CNAs employs such as karyotyping and FISH, CGH and array-CGH, these approaches have suffered from several inherent drawbacks including, rare mutations, low resolution, and difficulty in detecting novel and limited coverage for genome.

Newly developed of Next generation sequence enables simultaneous measurement of copy number at thousands of sites in a genome, more accurate estimation of copy numbers, higher coverage and resolution, accurate detection of breakpoints, and higher capability to identify new copy number alternations. The application of NGS, mainly through whole genome and whole-exome technologies, has produced an explosion in the context and complexity of cancer genomic alterations.

5. Conclusions

Liver tumors divided into two parts: benign and malignant. HCC is one of the most common types of malignant tumors. The early diagnosis of HCC is indispensable for patient survival, but it represents a very difficult task. To date, no evidence has accumulated that improves survival benefit with surveillance of high-risk patients. Current HCC screening methods include mainly serum AFP test, CT, MRI, and ultrasonography examination. The complicity due to screening methods, which have no clear evidence to improve survival of HCC patient. This concern present the urgent need for a sensitive, specific, and facile strategy for pre-diagnosis, early detection, and monitoring of HCC that would provide significant clinical benefit. Actually, there is a critical unmet medical need to discover novel biomarkers for early detection of HCC. Early detection and differential diagnosis is the key to the success of tumor resection and good prognosis.

Characterization and understanding of new genetic and epigenetic alterations, which are important to liver carcinogenesis, may help to realize the molecular pathogenesis of HCC, as well as providing new therapeutic targets for HCC treatment.

Next generation DNA sequencing has brought genome sequencing to clinical laboratories and genomic strategies. Comparing to traditional approaches, the applications of NGS seem almost endless, allowing for rapid progress in many fields related to the biological sciences. Resequencing of the human genome has performed to identify genes and regulatory elements involved in pathological processes.

NGS has many benefits including the ability to fully sequence large numbers of genes in a single test and simultaneously detect deletions, insertions, identify novel copy number alterations, translocations, and exome-wide base substitutions in all known cancer-related genes. The future of next-generation approaches holds great promise for a better integration of multiple molecular layers, eventually it is have more meaningful impact for clinical applications. These powerful technologies will provide more information about the development of HCC, which may help for better diagnosis and prognosis of HCC. NGS promises to revolutionize cancer research, drug design, diagnosis and therapy.

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