CHAPTER-2

REVIEW OF LITERATURE
2.1 HISTORY

In the past decade numerous genome projects were successfully completed, which had the goal to sequence the whole genome of various organisms. On June 26, 2000, President Clinton and Prime Minister Tony Blair announced in a joint statement the completion of the first survey of the entire human genome – the genetic blueprint for human beings. The completion of the human genome certainly was the highlight of the genomic era. This landmark achievement promised to lead to a new era of molecular medicine, an era that would bring new ways to prevent, diagnose, treat and cure disease. Specifically, scientists would now be able to use the human genome to alert patients that they are at risk for certain diseases, reliably predict the course of disease, precisely diagnose disease and ensure the most effective treatment is used and develop new treatments based on molecular mechanisms.

![One Genome]

Figure 2.1 Caterpillar and butterfly of Orgyia antiqua L. Not only the genome, but in particular the proteins present – the proteome – determine the appearance and state of a biological organism (adapted from Lottspeich).

However, despite the great success and the progress made in the genetic field, the discovery did not keep its promise and many questions remain open. One of the big surprises was the relatively low number of about 20'000 human genes detected, only roughly three times more than present in yeast. Even though the human species appears much more complex than yeast, its difference is
of the genome” in 1990. Yet report suggests having its root in analytical biochemical techniques used for protein separation and in 1975 the first high resolution protein separations were achieved by two-dimensional gel electrophoresis. While in the late 70s the first computerized 2-D gel image analysis was developed to identify the differential changes. Further introduction of mass spectrometry ionization techniques have revolutionize this discipline and in mid-90s it became a main stream technique for protein identification by mostly replacing the Edman’s sequencing. The proteome is the entirety of proteins expressed in an organism, a cell, an organelle, but also in a tissue or a body fluid, including the modifications made to a particular set of proteins at a given time point under defined conditions. In contrast to the genome, the proteome is much more dynamic, strongly influenced by internal and external factors such as development, differentiation, temperature or stress and thus differs from cell to cell. Furthermore distinct genes are expressed in distinct cell types and many proteins may go through a wide variety of modifications that profoundly affect their activity. For example phosphorylation or glycosylation of certain amino acid residues can influence protein localization, stability, enzymatic activity and protein-protein interactions (Tischer et al., 2003; Proud, 2005; Restle et al., 2005; Spiriti et al., 2008).

2.3 FROM GENOME TO PROTEOME
Analysis of human genome suggests that humans have between 20,000 and 25,000 genes- only slightly larger than the approximately 19,000 genes in the genome of the worm Caenorhabditis elegans. Approximately 40% of the human genome encodes proteins with no known function (Edward et al., 2000). Although the DNA code provides the instructions for their amino acid sequence, there are estimated 1.5 million proteins. Assigning functions to these proteins and their interactions is one of the challenges of proteomics. The vast difference in complexity between humans and worms cannot be explained merely by the fact that humans have more genes.
Thus, the correlation between DNA sequence and proteins is low, reflecting alternate splicing as well as post-translational modification. The study of these modifications is another major component of proteomics. Proteomics may provide one means of explaining that gap, by showing how the interactions between proteins give rise to human complexity (Figure 2.3). The goal of proteomics is to obtain a more global and integrated view of biology by studying all the proteins of a cell rather than each one individually. The aim of proteomics is not only to identify all the proteins in a cell but also to create a complete three-dimensional (3-D) map of the cell indicating where proteins are located. Ultimately, proteome analysis is like taking a photograph of the expression pattern of modified proteins and their isoforms at a particular time.

2.4 TECHNIQUES FOR BIOMARKERS DISCOVERY
There are two main approaches to biomarker discovery: The genomic approach, which focuses on identifying genetic mutations or changes in gene expression on
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micro RNA levels and the proteomics approach, which mainly examines the difference in protein levels between cancers and benign conditions. The various techniques used in the discovery of proteomic biomarkers including 2D polyacrylamide gel electrophoresis (2D - PAGE) and isobaric Tag for Relative and Absolute Quantification (iTRAQ) in addition to common techniques used in the validation of biomarkers such as western blotting and enzyme-linked immunosorbent assay (ELISA).

Figure 2.4 The various discipline of proteomics technique
(www.pharmafocusasia.com/research_development/images/various_proteomics_technique.jpg)

The key to increasing the chances for the success in any proteomics experiment is to have a thorough understanding of the physiological model or disease process being studied, from which a hypothesis is formed that will drive the choice and selection of particular proteomic/analytical approach. A typical proteomics experiment (such as protein expression profiling) can be successfully
executed under the following categories: i) sample preparation and protein solubilisation, ii) separation of proteins by 2-DE, iii) protein detection, iv) 2-D image analysis and quantification, v) identification of selected proteins, vi) validation (Figure 2.4).

2.4.1 SAMPLE PREPARATION AND PROTEIN SOLUBILIZATION
The three fundamental steps in sample preparation are cell disruption, protein inactivation plus removal of interfering substances, and solubilization of proteins of choice. Additionally, in some cases, proteins need to be enriched or prefractionated. It is desirable that the sample preparation should be as simple and reproducible as possible (Gorg et al., 2004). Treatment of biological sample involves cell lysis or another disruptive step to yield a suspension of cells, organelles, or fragments of subcellular structures. Proteins are extracted from this suspension with a combination of chemicals (Herbert, 1999). Detergents (e.g. CHAPS, SDS and Tween) are used to aid solubilization of membrane proteins and their separation from liquids. Chaotropes (e.g. urea and guanidine) are added to solubilize hydrophobic proteins. Reductants (e.g. dithiothreitol and mercaptoethanol) help to prevent oxidation and break disulfide bonds. Enzymes (e.g. DNase and RNase) may be used to decompose contaminating nucleic acids, lipids, and carbohydrates. Protease inhibitors are added to prevent protein degradation by endogenous proteases.

2.4.2 TWO DIMENSIONAL GEL ELECTROPHORESIS
The predominant technology for protein separation and isolation is polyacrylamide gel electrophoresis. The use of polyacrylamide in electrophoretical separations was first introduced by Raymond and Weintraub in 1959. Though there have been promising alternative and new techniques introduced into proteomics 2-DE in combination of mass spectrometry and/or immunoblotting still remains the most widely used methodological approach in differential and quantitative proteome analyses (Graham et al., 2005). This technique involves the separation of complex mixtures of proteins first on the
basis of isoelectric point (pl) using isoelectric focusing (IEF) and then in a second dimension based on molecular mass. The proteins are separated by migration in a polyacrylamide gel. The combination of these two techniques produces resolution far exceeding that obtained in 1-DE. One of the greatest strengths of 2-DE is the ability to resolve proteins that have undergone some form of posttranslational modification. This resolution is possible in 2-DE because many types of protein modifications confer a difference in charge as well as a change in mass on the protein. By use of different gel staining techniques such as silver staining, coomassie blue stain, fluorescent dyes, or radiolabels, few thousands proteins can be visualized on a single gel (Merril et al., 1979; Patton, 2000). The latest innovation in 2-DE was the development of the differential in-gel electrophoresis (DIGE) technology. It is based on labeling proteins with fluorescent Cy2, Cy3, and Cy5 dyes. All of these dyes have identical Mr and pl but differ in excitation and emission wavelengths. Therefore, up to three different protein mixtures can be labelled individually but separated and analyzed on a single gel. After scanning one gel, three separate images are obtained.

2.4.2.1 IMAGE ANALYSIS
The traditional workflow in image analysis includes spot detection, matching of proteins to corresponding spots on a reference gel, background subtraction, and quantification. Stained gels scanned at different resolutions with laser densitometers, fluorescent imager, or other device. The data can be analyzed with software such as PD-Quest by Bio-Rad Laboratories (Hercules, Calif, USA) (Bergman et al., 2000), Melanie 3 by Gene Bio (Geneva, Switzerland), Image master 2D Elite by Amersham Biosciences, and De Cyder 2D Analysis by Amersham Biosciences (Buckinghamshire, UK) (Chakravarti et al., 2002). Ratio analysis is used to detect quantitative changes in proteins between two samples. 2DE is currently being adapted to high-throughput platforms (Lopez et al., 2000).
2.4.3 ISOBARIC TAG FOR RELATIVE AND ABSOLUTE QUANTIFICATION

Isobaric Tag for Relative and Absolute Quantification (iTRAQ) is a chemical labelling multiplexing technique, which quantifies the concentration of proteins using mass spectrometry (Wu et al., 2006; Shadforth et al., 2005; Tonack et al., 2009). iTRAQ coupled with electro-spray ionisation tandem mass spectrometry. The technique of iTRAQ-MS relies on the fact that proteins can be digested to a unique set of different tryptic peptides. The iTRAQ part of the technique consists of the digestion of proteins in a sample into their constituent peptides followed by the labelling of these peptides by isobaric tags. Each sample group (i.e. HCC and Control) is labelled with a different tag with a unique reporter group of a specific mass, which is released during mass spectrometry through collision-induced dissociation thus allowing the association of a peptide with a specific sample group. The detection part of iTRAQ-MS involves the vaporisation and ionisation of the labelled peptides through an electromagnetic field. The resulting trajectory data and mass-to-charge ratio data from the MS analysis can then be used to identify the protein origins of these peptides. The different tags allow the relative quantification of peptides between samples, for example, a given peptide labelled with the disease-specific tag could be four times more abundant than the same peptide labelled with the control specific tag, indicating that the peptide is more abundant in the disease than the controls. Mass spectrometry based methods such as iTRAQ offers the identification and quantification of numerous proteins in a single experiment. This is clearly advantageous for biomarker studies, where several potential biomarkers can be identified upon data comparison between the disease and control groups. However, there are two major drawbacks for this technique: iTRAQ requires a large amount of sample and the sample preparation stage may require weeks to complete especially in the case of serum, where abundant protein depletion is necessary. An acceptable solution to this problem is the use of pooled samples but this is not without its own disadvantages. Although pooled samples present an “average” profile for the disease group in question, this “average” is very susceptible to skewing by an outlier with unusually high or low expression of a
particular protein. Therefore, it is important to validate the results from pooled samples by other proteomic methods such as western blotting or ELISA.

2.4.4 PEPTIDE AND PROTEIN IDENTIFICATION

Due to its high sensitivity and high-throughput, mass spectrometry (MS) is the method of choice for the identification and accurate quantification of the proteins contained in complex sample mixtures. Almost 20 years ago, the development of two soft ionization techniques, electrospray ionization (ESI) (Keller et al., 2005) and matrix-assisted laser desorption/ionization (MALDI) (Fenn et al., 1989; Hillenkamp et al., 1991) enabled the mass spectrometric analysis of large biomolecules. In 2002, Fenn and Tanaka were awarded with the Nobel Prize for their achievements. Recent advances in MS based proteomics, specifically improved instrumentation, software tools for the analysis of proteomic data sets (Godoy et al., 2006) and emerging, more efficient data collection strategies (Schmidt et al., et al 2008), now routinely lead to the identification of hundreds to thousands of proteins in a single experiment. MS employs chemical fragmentation of a sample into charged particles (ions) and measures charge and mass of the resulting particles, the ratio of which is deduced by passing the particles through electric and magnetic fields. A mass spectrometer consists of three essential modules: an ion source that transforms the molecules into ionized fragments, a mass analyzer which sorts the ions by their mass to charge ratio (m/z) and a detector that measures the ion intensity and thus provides data for calculating the abundance of each ion fragment present (Figure 2.5). Mass spectrometers mainly differ in respect to the ionization method (ESI or MALDI) and mass analyzers used. The performance and characteristics of a mass spectrometer thus greatly depend on the instrumental setup. The mass accuracy, resolving power, sensitivity and dynamic range of the instrument thereby greatly influence the capability of peptide identification and quantification and detecting modifications thereof at high-throughput (Domon et al., 2006).
**Figure 2.5** schematic representations of various types of mass spectrometry (MS) experiments. (A) In MS mode only the first mass analyzer is used where the peptide ions are analyzed and a full mass spectrum is generated representing the peptides analyzed at a time. (B) The purpose of MS/MS experiment in proteomics is the generation of fragment ion spectra for the identification of the amino acid sequence of specific peptides. In this experiment, the first analyzer (MS1) is set to a value that selects one specific precursor ion at a time. The selected ion undergoes CID in the collision cell, and the resulting fragments are analyzed by the second analyzer by screening an extended mass range (MS2). This process is repeated for different precursors. (C) SRM consists of a series of short experiments in which one precursor ion and one specific fragment characteristic for that precursor are selected by MS1 and MS2, respectively. Typically, the instrument cycles through a series of transitions (precursor - fragment pair) and records the signal as a function of time (chromatographic elution). SRM is used for the detection of a specific analyte with known fragmentation properties in complex samples.
In electrophoresis-MS-based identification, proteins are first separated electrophoretically and excised from gels followed by proteolytical in-gel digestion e.g. with trypsin. The resulting peptides are analysed by MS and the proteins identified by database searches (Thiede et al., 2005) (Figure 2.6).

![Figure 2.6 The sequence of events during the processing by proteomic technology](www.bio.miami.edu/~cmallerv/255/255tech/mcb3.33a_2D.jpg)

### 2.5 PROTEOMICS IN CANCER BIOMARKERS - A VIEW TO NEAR FUTURE

Proteomics is the indispensable tool for biomarker(s) discovery that provides the better understanding of cancer progression as well as can help in its early detection. Due to the complex multifactorial nature and heterogeneity of the cancer syndrome it is not detected at early stage (Marrero, 2005; Posadas et al., 2005; Pang et al., 2008; Fan et al., 2009). To date, no effective treatment is available for advanced cancers, which remain a major cause of morbidity and mortality. Clearly, there is an urgent need to unravel novel biomarkers for early detection. Most of the functional information of the cancer-associated genes...
resides in the proteome. Assuming that the proteome, is the global representative of all biological process that takes place in the cancer cells, then the discovery of specific biomarkers in the midst of such biological complexity seem difficult in the absence of ultrahigh resolution analytical technique for quantitative measurement to tens to hundreds of thousands of components, and robust data acquisition and analysis techniques to efficiently and reliably process large datasets (Figure 2.7).

Figure 2.7 Application of proteomic to biomarker validation (www.landesbioscience.com/curie/images/chapters/heicolor.gif).

Proteomic encompasses the identification and quantitative analysis of differentially expressed protein relative to healthy tissues counterparts at different stages of the disease, from preneoplasia to neoplasia. Proteomic technologies can also be used to identify marker for cancer diagnosis, to monitor disease progression, and to identify therapeutic targets. Proteome is valuable tool
in the discovery of the biomarker because the proteome reflects both the intrinsic genetic makeup of the cells and impact of its immediate environment. It enables the identification or the state and stage specific protein. Research efforts are already underway to develop the technology necessary to compare specific protein profiles of the diseased versus non-diseased states, and treated versus non-treated cases. Current progress in proteomics has been largely due to recent development in mass spectrometry (MS)-based technologies. Particularly, techniques for the ionisation of protein and peptides, such as matrix-assisted laser desorption-ionisation (MALDI) and electrospray ionisation (ESI) combine with time-of-flight (TOF), as well as new hybrid mass spectrometer, has now proved as the tool of choice for protein characterization. MS has been helpful in the analysis of protein form cancer tissues. Screening for the multiple form of the molecular chaperone 14-3-3 protein in healthy breast epithelial cells and breast carcinomas a potential marker for the non-cancerous cells (Vercoutter et al., 2001). The 14-3-3 form was observed to be strongly down regulated in primary breast carcinoma and breast cancer cell lines relative to healthy breast epithelial cells using the MALDI-MS system, Yung et al. (2009) detected increase in the expression of nuclear matrix, redox and cytoskeletal proteins in breast carcinoma relative to benign tumors. SELDI is a method that provides protein profiles or pattern on a short period of time from a small starting sample, suggesting that molecular fingerprint may provide insight into changing protein expression form healthy to benign and pre-malignant to malignant lesion. A SELDI proteomic profiles for prostate, lung, ovarian and breast cancer have been evaluated and described recently. Yim (2006) analyzed the proteomics profiles of ursolic acid (UA) induced apoptosis in cervical carcinoma cells. More than 45 proteins showed significant changes > 2.5 fold in UA treated cell as compared to control cells. Identified proteins were mostly involved in apoptosis including caspase -3,-5, -7 fas (CD95) calpain and cyclin dependent kinase (CDK5). The expression data obtained with 2DE proteomics were strongly correlated with the western blot and RT-PCR methods. A deeper insight into the mechanism of action of paclitaxel (potent drug of natural origin) on cervical carcinoma cells by employing proteomic profiling with functional analysis using RNAi technology.
Paclitaxel treatment elevated mainly apoptosis-related, immune response-related and cell cycle check point-related proteins (Lee et al., 2005; Lee et al., 2009).

2.6 HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is a type of cancer that arises from the hepatocyte, which is the major cell type of the liver. HCC is the fifth most common cancer in the world with a 5 year survival rate of less than 5% and an incidence of at least one million new patients per year (Bruix et al., 2004). Although HCC is more common in Asia and Africa, its incidence has increased substantially in many western countries during last twenty years (Befeler et al., 2002; El-Serag, 2004). HCC is usually asymptomatic in the early stages and has a tendency for intravascular or intrabiliary invasion, even when the primary tumor is small (Fong et al., 2001). Usually, HCC symptoms appear at an advanced stage, so early diagnosis is critical for a good prognosis. Historically, only 10–20% of primary HCCs are found to be resectable at the time of diagnosis (Fong et al., 2001). HCC is associated with chronic liver injury, primarily chronic viral hepatitis and alcoholic liver disease (Bruix et al., 2004; Llovet et al., 2004). The highest incidence of HCC is found in areas where the hepatitis B (HBV) and hepatitis C virus (HCV) are endemic. In the case of HBV, it has been demonstrated that the relative risk of developing HCC is 25- to 35-fold greater in individuals with evidence of chronic infection compared to non-infected individuals (Wands, 2004). In chronic HCV, there are no estimates of relative risk, but the incidence of HCC in cirrhotic carriers of HCV may be as high as 5% per year, compared with 0.5% in HBV carriers (Bisceglie, 1995). About 80% of people with HCC have cirrhosis (Kamel and Bluemke, 2002). Accumulating report suggested the existence of a high level of p53 alterations in HCC and focussed on its roles in the pathogenesis and development, diagnosis and treatment, and therapeutic effects and prognosis of HCC (Attallah et al., 2003; Marotta et al., 2004; Guzman et al., 2005; Bai and Zhu, 2006; Breuhahn et al., 2006). Development of HCC is a multi-stage process, which is closely related to viral infection and chemical carcinogens. In the molecular aspect, dysregulation of pleiotropic growth factors, receptors and their downstream signaling pathway components represents a central pro-tumorogenic principle in human
hepatocarcinogenesis (Breuhahn et al., 2006; Juan et al., 2010) (Figure 2.8). p53 is one of the tumor relevant factors. Its inactivation by point mutations within its gene or allelic deletions as well as the complex formation of p53 with cellular or viral proteins is a common and crucial event in carcinogenesis. In the regions with dietary exposure to the fungal aflatoxin B1 (AFB1), a specific mutation at codon-249 of the p53 gene is detected in plasma of 30-47% of patients with HCC (Kimbi et al., 2005). AFB1 is a common HCC carcinogen. Elevated levels of aflatoxin B1-albumin adduct (AFB1-Alb) have been associated with an increased risk for HCC development. In vitro experiments have also shown that treatment of human liver cells with AFB1 leads to p53 mutations (Tong et al., 2006). When using transgenic mice, HCC development was accelerated upon AFB1 exposure, demonstrating the tumor-promoting role of mutated p53 (Tong et al., 2006). Furthermore, the DNA-binding domain of mouse p53 was replaced by human homologous segment to produce human p53 knock-in (Hupki) mice. After AFB1 injection for HCC formation, Hupki mice were found more susceptible to AFB1 than wild-type mice, suggesting that the humanized p53 was less effective than mouse p53 to prevent malignant transformation in mouse cellular environment (Tong et al., 2006).

Figure 2.8 Diagram showing mechanism of liver carcinogenesis.
Actually, inactivation of p53 by mutations and regional allelic deletions was found more frequently in tumors associated with HBV infection (Cougot et al., 2005) (Figure 2.9).

![Figure 2.9 Schematic illustration of the involvement of p53 in hepatocarcinogenesis. The development of HCC is a multi-stage process. Several extrinsic factors, such as aflatoxin, HBV, nutrition, alcohol, and trace elements, are thought to initiate or/and promote the hepatocarcinogenesis. Alteration of p53 status is an important intrinsic factor in this process. Inactivation of p53 by point mutation, allelic deletion, or complex formation with cellular or viral protein is a common and crucial event in the occurrence and progression of liver cancer (Guan et al., 2006).](image)

Many naturally occurring or synthetic chemicals to which humans were exposed via accidental contamination of food or water were also shown to induce liver cancer in experimental animals (Holmes, 2001), and such chemicals could cause G-to-T transversion at codon-249 of p53 gene (Kar et al., 1993). Other HCC-related agents, implicated in the development of HCC in correlation with p53, include nutrition (Mehta, 1995), alcohol consumption (Staib et al., 2003), vinyl chloride exposure (Weihrauch et al., 2000), oral infection (Sarin et al., 2001), oral contraceptive use (Benedetti et al., 1996), and some trace elements such as selenium (Wei et al., 2001; Irmak et al., 2003).
2.7 EPIDEMIOLOGY AND ETIOLOGY
According to the World Health Organization, cancer is a leading cause of death worldwide, accounting for 13% of all deaths. Liver cancer is the third leading cause of cancer-related death and approximately 75% to 80% of cases of HCC occur in Asia (Bosch et al., 2004; Kudo et al., 2010). In the USA, HCC is much less common than in other parts of the world and accounts for only about 16,000 or 2.9% of cancer deaths (www.cancer.org) (Figure 2.10).

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<tr>
<th>Continent</th>
<th>Men (396,364)</th>
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<td>North America</td>
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<td>Oceania</td>
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Figure 2.10 Liver cancers in the world (Curado et al., 2010; IARC Press).

In most Asian countries, HCC is ranked from number 1 to number 5 among the leading causes of death. In Mainland China and Taiwan, the incidence of HCC has been increasing in the past 30 years, but in Japan, the incidence has been relatively stable during that period (Chung et al., 2010). In Korea, particularly in the male population, the incidence of HCC decreased slightly in
the past 10 years. The primary etiological factor in Asia is hepatitis B. As exemplified by Korea, hepatitis B virus (HBV) accounts for 70–75% of HCC cases and hepatitis C virus (HCV) accounts for 10–15% (Han et al., 2007). In Hong Kong, 80% of HCC cases are caused by HBV and around 7% are caused by HCV. Japan is unique in the etiology of HCC in Asia because almost two-thirds of cases are caused by HCV and only 15% are related to HBV (Yu et al., 2000; Ikai et al., 2007; Kim et al., 2008; Chung et al., 2010). Taiwan appears to be in between. In the early 1980s, HBV was the dominant cause of HCC in Taiwan, accounting for 88% (Yu et al., 2000), but in the past 30 years, HCV increased significantly and now accounts for more than 30%. HBV remains the predominant cause, but because of a vaccination program that was started in 1984, Taiwanese younger than 25 years old have a carrier rate of around 1%. Thirty years from now, HBV-related HCC will decreased dramatically in Taiwan and in other countries that have adopted a nationwide HBV vaccination program (Yuen et al., 2009).

Regarding the age distribution of HCC, in all countries in which HBV is the dominant cause, the median age is around 55 years old. Statistics for Japan, which is characterized by HCV, show that the median age is about 10 years older. The cumulative life time risk of liver 0.88% in men and 0.42% in women; and increase in HCC has been driven by an increasing proportion of those patients developing cirrhosis over time (Wong et al., 2000). The overwhelming majority of HCC cases occur in patients with chronic liver disease (Simonetti et al., 1991). Cirrhosis of any cause can result in HCC but chronic viral hepatitis accounts for more than 80% of cases worldwide (Bosch et al., 2004). Among patients with cirrhosis due to hepatitis C, HCC is the first complication to develop in 27% of patients; is the most common complication; and is the main cause of death (44%) (Sangiovanni et al., 2006).

2.8 RISK FACTORS

Cirrhosis

Almost all HCC occurs in the setting of cirrhosis or advanced fibrosis. Therefore, any agents that induce of liver disease which further result in cirrhosis should be considered a potential risk factor for HCC (Snowberger et al., 2007).
Hepatitis B and C

Hepatitis B is one of an increasing number of human viruses, including HBV, HCV, Epstein-Barr virus, Kaposi sarcoma–associated herpesvirus, human papillomavirus, and human T-cell leukemia virus, which have been directly linked to carcinogenesis. The pathogenesis of HCC in patients with chronic HBV infection remains speculative, however, and it is apparent that no single mechanism is predominant. HBV DNA sequences can be integrated into liver tissue, and this occurs early during the course of infection (acute hepatitis) (Lugassy et al., 1987). Integration may result in host gene deletions, rearrangements, chromosome transpositions and instability (Brechot et al., 2004). Furthermore, integration can occur in genes encoding cell signaling and proliferation proteins. Some of these targets, such as the telomerase gene, are common, suggesting a common pathway in hepatocarcinogenesis. A significant number of tumor cells demonstrate integration of hepatitis B X (HBx) or truncated envelope (S or pre S2) sequences. HBx transactivates a number of cellular promoters and therefore might activate cell signaling pathways that regulate target gene expression and cell proliferation (Brechot et al., 2004). In addition to these HBV-specific pathways, inflammation and cirrhosis result in cell proliferation that can lead to gene instability and rearrangements, a mechanism that may be most important in non viral causes of cirrhosis. HCV currently explains about 30% of cases worldwide (Bosch et al., 2004). HCV is the most common cause of HCC in the USA, Europe, and Japan, accounting for 47%–49%, 56%, and 75% of cases, respectively (Bisceglie et al., 2003; Kiyosawa et al., 2004). In contrast to HBV, however, HCV is an RNA virus without reverse transcriptase activity and therefore does not integrate into the host genome. Nonetheless, several viral proteins have properties that make them suspects of interest. In particular, the HCV core protein impacts numerous cellular processes, including apoptosis, cell signaling, transcription activation, cell transformation, and immune response (Lai et al., 2000), while the E2 envelope protein and the nonstructural NS3 viral protease interfere with the activation of endogenous interferon, which may increase cell proliferation and inhibit host cancer surveillance (Taylor et al., 1999; Foy et al., 2003). However, alcohol
appears to be associated with HCC only as consequence of alcoholic liver disease, in particular cirrhosis. Direct data supporting alcohol use in the pathogenesis of HCC are lacking and the relationship, if any, is controversial (Simonetti et al., 1992; Fukushima et al., 2006). The risk of HCC is highest in patients with heavy alcohol use and cirrhosis due to chronic hepatitis C (Simonetti et al., 1992; Ikeda et al., 1998; Niederau et al., 1998; Aizawa et al., 2000). This suggests that perhaps the major impact of alcohol on HCC risk is its effect in accelerating fibrosis progression in patients with chronic hepatitis B and C. It is not known whether alcohol is directly carcinogenic for these tumors or acts as a cocarcinogen. Acetaldehyde, the main metabolite of alcohol, causes hepatocellular injury and is an important factor in causing increased oxidant stress, which damages DNA. Patients with genetic hemochromatosis have a risk of HCC that may be increased as much as 200-fold compared with that of the general population (Bradbear et al., 1985).

**Aflatoxin**

HCC has been associated with dietary exposure to aflatoxin in regions of the world, including sub-Saharan Africa and Asia, where fungal contamination of grain is common. This risk appears to be confined to patients with chronic HBV infection. HBsAg-positive patients who have detectable aflatoxin-albumin adducts in the urine have a significant increase in HCC risk compared with those who lack this marker (Yu et al., 1997).

**Diethyl-nitrosodiethylamine (DEN)**

Diethyl-nitrosodiethylamine (DEN) is one of the leading hepatocarcinogen of nitrosamine family. It form DNA carcinogen adducts in the liver and induce hepatic cancer (Yang et al., 2004; Mihael et al., 2010). DEN mostly found in various food stuff like, cheese, dried fish, cured meat, alcoholic beverages etc, also found in ground water having high level of nitrates (Sullivan et al., 1991; Brown 1999; Farazi and Depinho, 2006; Jemal et al., 2009. DEN treatment increases the permeability of mitochondrial transitional pore, this action leads to increased production of H$_2$O$_2$ through mitochondria and initiate peroxidative
stress (Malik et al., 2012). It can also activate the enzyme cytochrome P450 which produces reactive electrophilic compounds, that has ability to increases oxidative stress and casuse cytotoxicity and carcinogenicity (Zimmerman et al., 1993).

2-Acetylaminofluorene (2-AAF)
2-Nitrosoflourine, a metabolite of 2-AAF, has been shown to induce redox cycling leading to superoxide production capable of reacting to DNA, disrupting the cell membrane integrity and causing lipid peroxidation. 2-AAF has also been reported to induce tumorigenesis in rat liver by stimulation of cell proliferation (Tiwawech et al., 1991; Lei et al., 2001). It has been investigate that adminstration of 2AAF in rats can effect histone lysine methylation patterns, via trimethylation at lysine 9 and lysine 27 residue of histone H3 in the promoter regions of Rassf1a gene, and other tumor suppressor gene like p16 (INK4a), Socs1, Cdh1, and Cx26 gene, also have ability to hypermethylation of early Rassf1a and p16 (INK4a) promoter CpG island. These alterations clearly show imbalance between cell proliferation and apoptosis (Calvisi et al., 2012). Lower dose of 2-AAF (50 mg/kg body weight, i.p.) has ability to enhances hepatic lipid peroxidation, hepatic glutathione content depletion, glutathione peroxidase, glutathione reductase, catalase, and glutathione-s-transferase (Sehrawat and Sultana, 2006). Some time small amount of 2-acetylaminofluorene (2.24 or 22.4mg/kg) for long time (3 times per week for 31days) can produce hepatocellular carcinogenesis via AAF induced DNA adducts (Iatropoulos et al., 2012).

2.9 PATHOGENESIS
The mechanisms by which viruses and/or hepatic fibrosis lead to HCC are not entirely clear. It is, however, clear that two general processes are involved. The first is not specific to a particular etiology but rather involves a wide variety of dysregulated mechanisms that result from hepatic inflammation, necrosis, and regeneration. Signaling dysregulation that favors tumor growth involves many different factors such as insulin-like growth factors, hepatocyte growth factor, and the Wingless (Wnt)/betacatenin signaling axis (Breuhahn et al., 2006; Farazi and De-Pinho, 2006). These factors are up-regulated in HCC, but it is difficult to discern the relative contributions of each since their functions and effects often
overlap and alterations accumulate over time. As a result, growth may accelerate and signals may become unresponsive to inhibitory factors. Recently, gene expression profiles of tumors were analyzed in 103 HCV-related HCC (Chiang et al., 2008). Three patterns were associated with pathway activation (Wnt/beta-catenin, tyrosine kinase receptor activation, and interferon response over-expression) while another was associated with over-expression of several proliferative and tumor activation factors associated with chromosome 7 polysomy (Cheung et al., 2008). Additionally, tumor cells may evade normal apoptosis mechanisms and facilitate angiogenesis (Breuhahn et al., 2006). In the case of hepatitis B, integration of viral DNA into the host genome might lead to chromosomal instability and mutations in normal proliferation regulatory factors, including tumor suppressor genes such as p53 (Koike et al., 2005). Thus, it would be the cumulative effect of these alterations that would result in HCC (Figure 2.11).

![Multi-Step Pathogenesis Diagram](image-url)
Other cofactors might provide the second hit. For example, aflatoxin exposure may also result in p53 mutations, DEN forms DNA adduct in the liver and induce hepatic cancer, vinyl chloride causes K-ras mutations, and hepatic adenomas are associated with hepatocyte nuclear factor 1 mutations (Koike et al., 2005). The pattern of these dysregulatory processes is probably responsible for the wide variation in the clinical and histological presentation of this malignancy.

2.10 DIAGNOSIS

Alpha-fetoprotein (AFP)

AFP is FDA approved biomarker for HCC, a glycoprotein produced in foetal liver whose production falls after birth, and repressed in adults. AFP has been used widely as a serum marker for HCC for decades (Chaerkady et al., 2008). It was considered to be sensitive and specific for HCC until the introduction of sensitive imaging techniques such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) and it is now apparent that the test has limited specificity and the presence of chronic inflammatory liver diseases such as hepatitis its level raise to more than 100 ng/mL. Though marked elevation of AFP has prognostic value. Total AFP consists of three glycoforms that are according to their binding affinity to the lectin Lens culinaris agglutinin (Zhou et al., 2006). These glycoforms include AFP-L1 (non binding), which accounts for AFP elevations from non malignant hepatic disease, and AFP-L3 (highly bound), which is the prevalent form in HCC patients with elevated AFP. AFP-L3 suffers from the same problems with sensitivity as undifferentiated AFP, but elevations are more specific. As with the undifferentiated protein, high levels of AFP-L3 are associated with advanced tumor (Khien et al., 2001).

Des-gamma-carboxy prothrombin (DCP)

DCP is also FDA approved protein that is induced in the absence of vitamin K and is an aberrant byproduct from disturbed carboxylation during the formation of thrombin (Marrero et al., 2003). DCP acts as an autologous mitogen for HCC cell lines. It is elevated in 50% to 80% of patients with HCC and does not correlate
with AFP elevation. In fact, it appears to be more specific for HCC than AFP and is less often elevated in cirrhotic patients without HCC (Kasahara et al., 1993; Marrero et al., 2003). Thus, the combination of DCP with AFP or AFP-L3 might be even more sensitive and specific (Kasahara et al., 1993; Zinkin et al., 2008). Accept of these 2 FDA approved biomarker numerous other genes and proteins that are expressed in patients with HCC might prove useful as clinical markers but are not well studied to date. These include alpha-1-fucosidase, AFP mRNA, gamma-glutamyl transferase mRNA, human telomerase reverse transcriptase mRNA, vascular endothelial growth factor and tumor-specific growth factor. Glypican-3 is a heparin sulfate proteoglycan and overexpressed in HCC condition (Liovet et al., 2006). It is detectable in the serum of 40% to 50% of HCC patients, including a third of those without detectable AFP (Capurro et al., 2003). It is also reported to be present in patients with some other tumors, including germ cell tumors and gastric carcinoma. The ability of abdominal imaging to detect HCC has improved dramatically over the last 2 decades, and the methods described below have generally replaced more invasive procedures (e.g., angiography, exploratory laparotomy, and percutaneous biopsy) as the preferred tools to identify hepatic tumors. Despite this progress, however, ultrasound, CT, and MRI remain variably insensitive for detecting HCC, particularly with tumors <2 cm in diameter (Baron et al., 2004; Taouli et al., 2004; Colli et al., 2006; Snowberger et al., 2007). New hardware and software technology that was introduced for CT and MRI in 2000 improved the sensitivity of both modalities. CT scanning identifies about 70% and ultrasound about 60% (Snowberger et al., 2007). Though these are good at estimating tumor size but none are very accurate in documenting the total number of lesions present; this is likely related to size, different imaging characteristics of tumors, and location. Standard ultrasonography is widely available, relatively inexpensive, and easily performed. Thus, it has become the most commonly utilized imaging modality for detection of hepatic masses. Furthermore, it is able to confidently distinguish some benign lesions such as cysts. CT and MRI rely on examination of tissue characteristics during and after arterial contrast enhancement. Typically, images are obtained during the early arterial phase, the late arterial (portal) phase, and a delayed
CHAPTER 2

REVIEW OF LITERATURE

phase (90–120 seconds post contrast). HCC demonstrates similar characteristics with both methods. MRI is more sensitive and provides more information than CT scanning. However, some lesions not detected by MRI may be found on CT. Thus, both modalities should be used when the index of suspicion for HCC is high, such as when the AFP level is very elevated. As with other imaging methods, the sensitivity of MRI is less for smaller lesions (Tanimoto et al., 2006). Positron emission tomography (PET) scanning is based upon the ability of certain markers to image molecular events that result from the over-expression of a gene that produces a specific messenger RNA. 18F-fluoro-2-deoxy-d-glucose (18F-FDG) is the most commonly used marker employed in PET scanning and is capable of detecting a difference in glucose-regulating mechanisms between some tumors and normal tissue. In liver, type 1 glucose transporter is most common in cholangiocarcinoma, while a rate-limiting glycolytic enzyme, hexokinase type II, is more common in HCC (Lee et al., 2005). While FDG-PET scanning is an excellent tool for cholangiocarcinoma, it is not particularly accurate for detecting HCC, with a reported sensitivity of about 50%. PET scanning might be helpful in evaluating patients with rising AFP levels without tumor detectable by other imaging techniques. The role of biopsy in confirming HCC is controversial (Durand et al., 2003; Bialecki et al., 2006). While biopsy was often required for definitive diagnosis before the advent of more sensitive and specific imaging tests, it is less often necessary today. Biopsy can be performed if the diagnosis remains in question and the result will influence the treatment plan. In such cases, the result of the biopsy is helpful if it is positive. Negative fine-needle aspirate results should be interpreted with caution. The risk of tumor seeding of the biopsy track is extremely low (Schotman et al., 1999).

2.11 TREATMENT AND PREVENTION

Many potential options for treating the HCC are now available. These are including the surgery, systemic chemotherapy, loco-regional treatment and symptomatic relief. Of all these surgery is proven to be good. The feasibility of liver resections is very low and the reasons for this include extensive local disease, presence of extra-hepatic disease. Chemoemoblation is the treatment
of an already developed cancerous tumor by directly injecting anticancer drugs (chemotherapy) into the blood vessels feeding the tumor. Chemoembolization is not a cure, but rather a treatment that reduces the severity of symptoms, temporarily improving the quality of life and potentially extending survival. Presently, chemoembolization is primarily used for the treatment of liver tumors which, because of size and location, are often inoperable. The procedure is based on stopping tumor growth by restricting the blood supply to the tumor while at the same time directing a large dose of medication specifically into the tumor. This procedure permits a high concentration of anticancer drugs to be administered into and remain in the tumor for a long period of time, thereby increasing its effectiveness. This directed method of delivery is called an "embolus" and is achieved by use of a catheter tube. This embolus approach allows the ability to focus treatment on the cancerous cells while protecting the rest of the body from chemotherapy exposure (Figure 2.12).

![Figure 2.12 Diagrams showing anticancer drugs directly injects (chemotherapy) into the blood vessels feeding the tumor through catheter tube.](image)

Researchers are now focusing on for the development of better method to detect the HCC at an early stage to allow the performance of curative surgery. Proteomic analysis of HCC has developed hope for the identification of novel
diagnostic biomarker and disease specific associated proteins that are potential therapeutic target in the treatment of HCC.

Cancer prevention is becoming a novel approach for cancer control. Common prevention approach include avoiding exposure to cancer causing agents, enhancing host defense mechanisms against cancer, modifying life style and chemoprevention. In the modern world, unfortunately, another evaluation becomes important in deciding what type of treatment to pursue: the financial one. While many of the techniques described above are effective in some patients, they are not necessarily always covered by insurance plans. Costs of the machines and drugs can be prohibitive to individuals: radioembolization can cost more than $90,000 for a single treatment; sorafenib is more than $5,000 for a month of therapy. This can make individual and institutional decisions even more heart-wrenching than usual on a personal level. At a societal level, these kinds of costs associated with treating this cancer makes it even more crucial to find ways to avoid developing it in the first place. Theoretically, hepatoma should be an almost entirely preventable disease. Hepatitis, alcohol abuse, and obesity could all be avoided through social, medical, and lifestyle changes. Some of this has already been attempted around the world, so there is cause for optimism. For instance, children in Taiwan have been immunized against hepatitis B since 1984. This has led, so far, to a 70% decrease in the rate of teenagers developing hepatoma. In the United States, where the incidence is already much lower than it is in Asia, hepatoma due to hepatitis B has fallen by half since immunization began. While there is not yet a vaccine against hepatitis C, this is a much easier virus to avoid now that blood products are being screened and people are more aware of preventing infection from used needles. Once someone is infected, treatment with the drug interferon can reduce the chance of developing hepatoma dramatically. Diabetes and obesity, clearly, can be reduced by modifications in diet and lifestyle, as difficult as that obviously continues to be in our society.
2.12 BIOMARKERS & ITS NEED FOR EARLY DIAGNOSIS OF OF HCC

A biomarker is defined by the National Institute of Health (BDWG 2001) as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic process, pathogenic process, or pharmacologic responses to a therapeutic intervention". The abundance or scarcity of cancer biomarkers in cancer relative to non-cancer conditions (e.g. inflammatory diseases and in health) may be an indication of changes to cellular biology in carcinogenesis. Therefore, it is the aim of cancer biomarker studies to identify these differentially expressed molecules and to assess their clinical usefulness as a screening, diagnostic, and/or prognostic modality for cancer. There are many samples, which can be used for the discovery of biomarkers including blood derivatives, pancreatic juice, tissue, saliva, and urine (Tonack et al., 2009). Of these, blood plasma or serum is most widely used in biomarker studies because they are readily accessible, minimally invasive to collect, generally acceptable to patients, and are potentially rich sources for most types of biomarkers (Tonack et al., 2009).

As previously discussed, the differential diagnosis of liver cancer is based entirely on non-specific symptoms, signs, and first-line investigation findings. Moreover, under the current recommendations, patients suspected of having liver cancer will undergo a series of relatively invasive procedures including radio-frequency ablation (RFA), microwave ablation (MWA) and transcatheter arterial chemoembolization (TACE) and biopsy. Clearly, there is a need for novel, accurate, and less invasive methods for the diagnosis of liver cancer such as a blood or serum-based protein biomarker. Science Daily (Aug 9'2007) – "Cancer of the liver is very difficult to detect, and it is a major cause of death in Asia and Africa, with rising incidence in Western countries as well." For the detection of HCC, only one marker (AFP) is generally used. However, this marker has a low specificity and is frequently inadequate because of false-positive results. Aside from the fact that a blood/serum-based biomarker would be less invasive compared to current diagnostic techniques, there are two other major advantages: the number of patients undergoing unnecessary invasive investigations and the time required to reach a diagnosis of HCC would be greatly
reduced. There is also the possibility that biomarkers can be employed to detect the presence of early liver cancer in otherwise asymptomatic individuals. Subsequently, this would mean that patients with HCC are diagnosed earlier thereby increasing their chances of having operable disease and therefore improving the prognosis.

2.13 CURRENTLY AVAILABLE BIOMARKERS OF HCC

Most proteomics studies on HCC are aimed at the discovery of new biomarkers. Identification of liver cancer biomarkers is one of the most rapidly advancing fields in clinical diagnostics. The most widely used biomarker for diagnosis of HCC is AFP that is present in the serum. A number of protein markers for diagnosis and prognosis of HCC have been reported (Table 1). These biomarkers include metabolic enzymes, proteins involved in calcium homeostasis, cytoskeleton and tumor suppression factors, and proteins that can increase cell’s resistance to apoptosis. New potential biomarkers were continuously discovered from HCC cell lines, tissues and serum. A few clinically approved liver cancer biomarkers (Table 1) are available for early detection or for successful monitoring of treatment and relapses. Early detection of HCC is critical in cancer control. However, none of the biomarkers listed in Table 1 has adequate sensitivity, specificity, and predictive value for population screening (Pei et al., 2009). So there is urgent need to unravel novel biomarker for detection of HCC.
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