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## Methylation Markers in Hepatocellular Carcinoma

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# **METHYLATION MARKERS IN HEPATOCELLULAR CARCINOMA**

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# **METHYLATION MARKERS IN HEPATOCELLULAR CARCINOMA**

**AVINASH GAVICHARLA**

## **ABSTRACT**

Hepatocellular Carcinoma is one of the major causes for mortality in the world and is usually asymptomatic until late stage decreasing the survival rate. There is an increase of incidence every year and current diagnostic and prognostic bio markers like AFP have their limitations in the wider context. Therefore, it is important to develop novel biomarkers.

The aim of the Research is to understand the cytosine methylation and investigate the role of the p16 and SLIT-2 genes as biomarkers in Hepatocellular Carcinoma. The methylation profiles of the genes were assessed on bisulfite modified DNA samples with the aid of Real time PCR. The specificity and sensitivity of the genes were calculated using the Standard Curve Analysis and specificity was determined by the ability to differentiate between methylated and unmethylated DNA. The sensitivity was determined by using different percentages of methylated and unmethylated DNA. This study identifies SLIT-2 as a potential biomarker and show the role of DNA methylation as an important part of hepato-carcinogenesis and its importance as a Biomarker.

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## **LIST OF ABBREVIATIONS**

HCC-Hepatocellular Carcinoma

DNA-Deoxyribonucleic acid

mRNA - Messenger Ribonucleic acid

PCR-Polymerase Chain Reaction

HPLC-High Pressure Liquid Chromatography

## **CHAPTER I**

### **INTRODUCTION**

#### **1.1 Epigenetics**

The term “Epi” means “on top off,” which explains the word “epigenetics” as another layer of information or a secondary layer of information on top of the genetic code. Epigenetics is nothing but the study of stable heritable changes in the genotype or the cellular phenotype which alters the final outcome without changing the underlying DNA sequences. Epigenetic studies are the studies of covalent and non-covalent modifications of DNA and the relative mechanisms which affect the overall chromatin structure. However, two important features distinguish the genetic alterations from the epigenetic changes namely, the gradual appearance and the reversibility of the epigenetic events. These two important features enables the epigenetic alterations a target for the therapeutic intervention and development of the strategies to prevent them.

The epigenetic information that can be inherited by the chromosomes can be categorized into three aspects namely (1), DNA methylation which occurs at the fifth carbon position of the cytosine pyrimidine ring with the addition of a methyl group via a

methyltransferase enzyme usually at the CpG dinucleotides, Epigenetic alterations involving the Ribonucleic acid in the form of non-coding RNA or RNA interference and lastly histone post translational modifications like acetylation, methylation, ubiquitination which occur at the N-terminal part of the tail of the histones which form a key part of the mechanisms and any deregulations of them leads to malignancies.

## **1.2 DNA Methylation**

DNA Methylation is the covalent chemical modification resulting in addition of methyl group at 5th carbon position of the cytosine ring. DNA methylation is a naturally occurring event in both prokaryotes and eukaryotes. In prokaryotes, they majorly play a role of protection of the host DNA from restriction endonucleases produced to destroy foreign DNA. However, they have a complicated role in eukaryotes like gene imprinting, X chromosome Inactivation, embryonic development and cell cycle regulation. When we consider the classic view of DNA methylation, it varies according to the developmental stages namely pre and post developmental processes. The former pattern associates the methylation processes into active or passive methylation whereas the latter involves its maintenance based on DNMT's (2). DNA methylation errors are potentially reversible and as a result aid in Pharmacological changes.

### **1.2.1 DNA methylation and Carcinogenesis**

Aberrant changes in the DNA methylation promote disease state. Aberrant DNA methylation is found in two distinct forms namely hyper methylation and DNA hypomethylation. Hyper methylation is usually associated with the gene inactivation

involving CpG islands and hypo methylation involving repeated DNA sequences of nuclear elements (3). These two processes are independent processes (4).

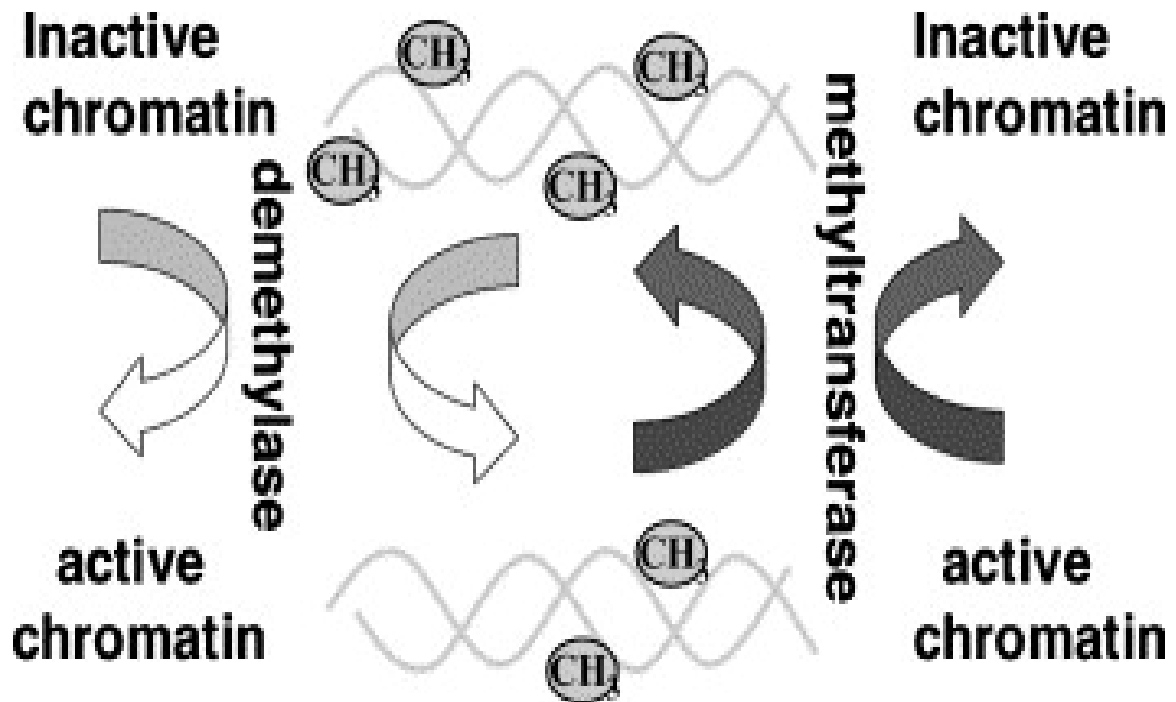
#### **1.2.1.1 Hyper methylation in Cancer**

It is one of the most studied epigenetic changes in cancer. DNA hyper methylation typically occurs in the CpG islands. The promoter site and transcription site are included within the CpG islands and when hyper methylation occurs at these islands, the expression of gene is totally repressed leading to gene inactivation (5).

#### **CpG Islands**

DNA is made up of four bases namely Adenine, Guanine, Cytosine and Thymine. Among these four bases, the numbers of dinucleotide combinations possible are 16 (6). Out of which, the cytosine methylation occurs in 5'CG 3' dinucleotide combination. Based on the percentage of these combinations, the frequency of these dinucleotides should be 10% but only occur around 5% (7). Methylation isn't uniform through the human genome, containing both methylated and unmethylated segments. Unlike the rest of the genome, CpG islands are interspaced on an average of 100kb and ranging up to 5kb are smaller regions of unmethylated DNA which are GC rich, Guanine Adenine content above 0.5 and having distinctive properties without any suppression of the occurrence of dinucleotide CpG. It is known that human genome contains around 29000 CpG islands and is associated with about 60% of mammalian genes, mostly in promoter and first exon region of genes. In normal, healthy tissues CpG islands are unmethylated but these are methylated to various extents in cancer (8).

There are different protective mechanisms that prevent the hyper methylation at these CpG islands like demethylation, changes in chromatin structure, active transcription and timing of replication (6).



**Figure 1.1: The methylation state of a gene is equilibrium of methylation and demethylation reactions (9).**

As the figure 1.1 indicates, DNA methylation is a reversible reaction and the active chromatin recruits DNA demethylases, the enzymes responsible for DNA demethylation and inactive chromatin recruits DNMT's. Therefore, if the chromatin structure is maintained DNA methylation is maintained. Hyper methylation is region specific and the

local changes around this specific region of genes inhibit them from interacting with the demethylases, protecting them from demethylation.

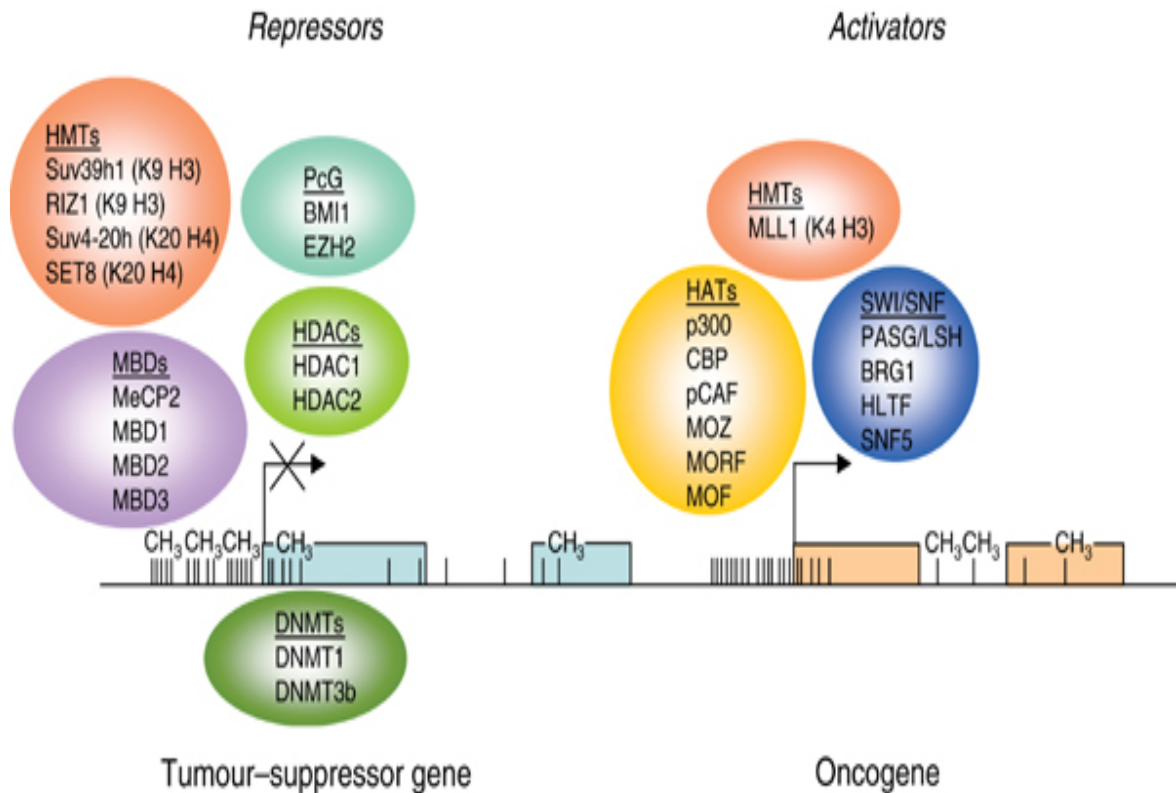
#### **1.2.1.2 Global Hypo methylation in Cancer**

Global DNA hypo methylation was the first identified epigenetic event in cancer. Hypo methylation is the loss of methylation and this loss is quite predominantly seen as they are quantitative in nature and affect a variety of repetitive sequences which are dispersed throughout the genome. Usually, the pericentric regions of chromosome 1-16 are hypo methylated, with most cases occurring in regions with sparsely distributed CG sequences. Hypo methylation plays a role in cancer by various mechanisms like instability of the chromosome (10), activation of the long interspread nuclear elements called retrotransposons in the genome (11). Hypo methylation of these transposons, which are usually latent, may lead to oncogenesis by transcriptional activation and sometimes may express themselves on the adjacent genes (12).

### **1.3 DNA Methylation Machinery**

Based on the methylation patterns of CpG dinucleotides on both strands, DNA methylation can be either Denovo Methylation or Maintenance Methylation. Denovo Methylation occurs, when the CpG dinucleotides on both the strands are unmethylated and has an important role in cell growth, differentiation and tumor genesis. Maintenance methylation occurs when CpG dinucleotides on only one strand are methylated (9). It mainly copies the DNA methylation patterns but does not introduce any different methylation patterns.





**Figure 1.2: Epigenetics provides a new generation of oncogene and tumor-suppressor genes**

As the figure 1.2 suggests, DNA methylation machinery can be classified into two ways namely, transcriptional repressor machinery to the corresponding promoter CpG island which contribute to tumorigenesis by silencing of TSG's and the Transcriptional Activation machinery for the active expression of the oncogene.

Transcriptional Repressor Machinery include DNA methyl transferases, Methyl CpG Binding proteins, histone methyltransferases for lysine 9 of histone H3 (HMT K9 H3), histone deacetylases and polycomb. Transcriptional Activator machinery includes HAT's, HMT's and others. Of these, epigenetic machinery DNMT's, MBD's and HAT's play an important role in DNA methylation.

### **1.3.1 DNA Methyltransferases (DNMTs)**

DNA methyl transferases are the enzymes that catalyze the transfer of the methyl group from the methyl donor S-adenosyl methionine onto the DNA and release S-adenosyl homocysteine (13). DNMTs are the critical proteins involved in establishing proper control of epigenetic information. In mammals, four different types of DNMTs are seen DNMT1, DNMT2, DNMT3a and DNMT3b. The balance of DNMT's is very important to prevent cell transformation. The DNMT's in conjugation with accessory proteins like DNMT3L are mainly responsible for DNA methylation at various stages like embryogenesis and development of somatic tissue (14).

DNMT 1 is the most abundantly found among DNA methyl transferases. It is useful in maintaining the post replicative DNA methylation patterns. It is useful for maintaining the methylation pattern during cell division. The DNA Methylation patterns are passed on from the parental strand to daughter cells and DNMT1 maintains this maintenance methylation (15). It also plays an important part in Denovo methylation (16). They maintain gene silencing in cancer cells, by cooperative interactions between DNMTs especially DNMT1 and DNMT3b. DNMT 2, is not involved much in DNA methylation. However, it can contribute to cancer through different pathways related to RNA. DNMT3b is mainly involved in Denovo methylation .It is also a part of several complexes. It inhibits gene expression by forming a transcription repressive complex. DNMT3B leads to demethylation when it is deleted and with DNMT1 and maintain the global methylation (17).

### **1.3.2 Methyl-CpG binding domain proteins**

MBD proteins are considered interpreters of DNA methylation. They are important between DNA methylation and genes involved in histone modifications. All of them bind to the CpG sites with the exception of MBD3 (18). Twelve different types of Methyl CpG proteins have been identified of which MBD1, MBD2, MBD3, MBD4 and MeCP2 are the most important members (17). They play a role in the pathophysiology by a number of mechanisms. They associate with the CpG island promoters of the Tumor suppressor genes and lead to transcriptional silencing. MBD4 is different when compared to other MBDs as it is a thymine glycolase and it helps in DNA repair protein, as the Glycolase domain removes thymidine from T: G mismatches (19).

### **1.3.3 Histone Modification Enzymes**

Histones are used to store the epigenetic information through modifications of the amino terminal protruding tails. These modifications generally include acetylation, phosphorylation, and methylation. Histone Modification Enzymes involved are histone acetyl transferases (HATs), Histone methyltransferases (HMTs) and histone deacetylases (HDACs). HATs are involved in the Acetylation of histone lysine's and HMTs aid in the deacetylation.

## **CHAPTER II**

### **HEPATOCELLULAR CARCINOMA**

#### **2.1 Introduction**

Primary Liver cancer can be distinguished into three main types namely Hepatocellular Carcinoma which occurs from Hepatocytes, Duct cell Carcinoma and cholangiohepatoma which occurs from intrahepatic bile ducts. Clinically there is not much difference between them since they spread throughout the liver (20). Primary Liver Cancer is unique when compared to all the malignancies because of its worldwide distribution, association with cirrhosis, spontaneity of the tumor and inherent difficulties (21). Primary Liver Cancer is the fifth most common cancer in the world and second rated cancer with most deaths (22). Hepatocytes, the parenchymatous cells which are chiefly responsible for its functioning and their uncontrolled division leads to primary liver cancer in most cases. Hepatocellular Carcinoma is the predominant type of liver cancer and also called as hepatoma based on its occurrence and it accounts for about 85-90% primary liver cancers (23). HCC is the fifth most common cancer in men and eighth most common in women. Hepatocellular Carcinoma is usually asymptomatic, with Cirrhosis being the major

condition present in about 80-90% of them and by the time of diagnosis it is usually advanced leaving with a 5 year survival rate. Hepatocellular Carcinoma have different growth patterns with some starting as single tumor and grows large before spreading and others appear as small spots in various areas. It is very important to diagnosis HCC early to improve the survival rate.

## **2.2 Epidemiology of Hepatocellular Carcinoma:**

Several epidemiological factors like age, sex, ethnicity, distribution of risk factors influence HCC. Geographically speaking, greater number of the cases of HCC is found either in sub-Saharan or Asian populations. In United States, the rates of incidence increased at a two-fold rate between 1985 and 2002. When you compare ethnicity among different regions, Asians are twice susceptible to HCC than Americans. This might depend on the differences in attainment of the risk factors of HCC. The age is an interdependent epidemiological factor based on various factors like sex, age of infection and so on. Hepatitis B Virus usually infects at younger age when compared to HCV. Throughout the world, age of incidence of HCC in men is approximately 5 years lower when compared to that of women. Sex of an individual highly influences the incidence rate because, throughout the ratio of incidence between male and female is either two fold or four fold with male dominating. This again might be due to various factors like exposure to viral factors, alcoholism and smoking with largest difference in ratios seen in European populations. Studies conducted state that men have higher BMI and higher level of androgenic hormones might be a reason for higher incidence of HCC. HBV and HCV are the two most dominant risk factors in HCC. However their distribution varies

from place to place. Globally, HBV risk factor is more dominant. In places like Japan HCV show greater dominance than HBV.

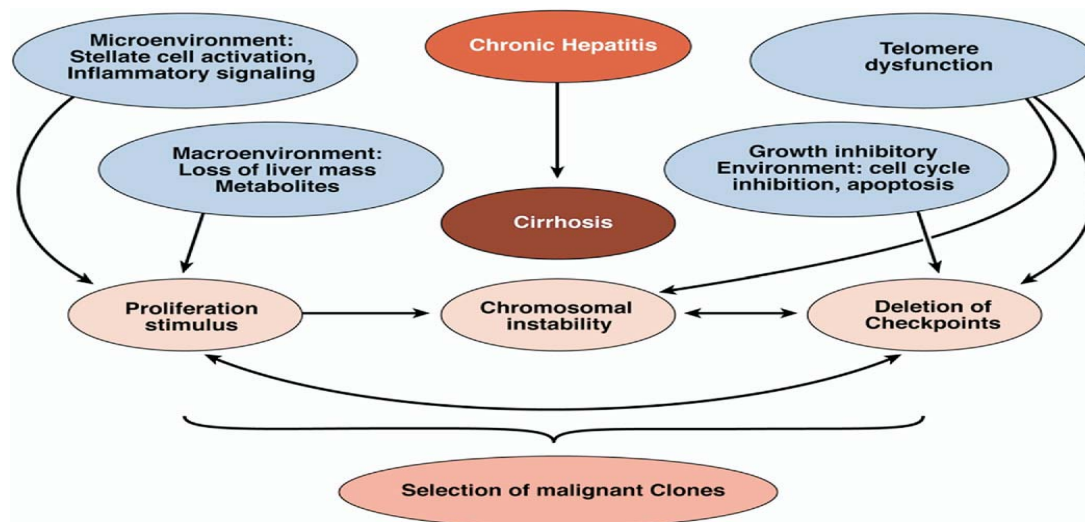
### **2.3 Risk Factors for Hepatocellular Carcinoma**

The risk factors of HCC can broadly be categorized into pathological factors, physiological factors, exposure to environmental toxins and dietary factors (23). The physiological factors like geography, age and sex are mostly patterns of the pathological factors mostly HCV, HBV and alcohol liver disease (24). The physiological factors for HCC incidence increase with age and males have greater chance of Incidence than females. The pathological factors can be categorized into Hepatitis B virus and Hepatitis C virus. Liver Cirrhosis can be considered to be the main precursor because about 70% have HBV related Cirrhosis when compared to the 20% HCV related Cirrhosis (25). Various studies show that incidence of HCC in patients with chronic Hepatitis or Cirrhosis is greater when compared to the patients with normal transaminases (26).HCV does not play a direct role in HCC. However it leads to development of Cancer through Cirrhosis. Exposure to environmental Toxins like Aflatoxin, which is a product of *Aspergillus* fungus, on the legume crops is less developing countries and on dietary intake of them leads to development of HCC (27) (28). Alcohol does not have any particular mutagenic properties. Effect of Consumption of Alcohol is usually dose dependent and leading to HCC through Cirrhosis. It was proved in a Cirrhosis study that chances of HCC are 13 times greater in people consuming alcohol when compared to people who stay away from it (29). Smoking is also an important factor in HCC, with studies showing a contribution of about 50% in HCC (30). Few of the rarer factors which influence HCC are hemochromatosis which is a genetic factor and few metabolic diseases

like Diabetes type-II, glycogen storage disease type 1 and alpha-1 antitrypsin deficiency (31).

## 2.4 Molecular Mechanisms of Hepato- Carcinogenesis

The pathophysiology of HCC is mainly based upon cirrhosis. Different molecular mechanisms inhibit the regenerative capacity of the liver at cirrhotic stage and these mechanisms tend to increase the rate of carcinogenesis at cirrhotic stage. They can be classified into cell intrinsic alterations and cell extrinsic alterations (23). Cell intrinsic alterations involved are the shortening of the length of telomere which restricts the proliferation of hepatocytes (32) (33), thereby leading to induction of chromosomal fusion by activating DNA repair pathways (34) (35). Cell extrinsic alterations involved mainly are the decrease in the proliferation of the primary liver cells and changes in the micro and macro environment, thereby promoting risk of cancer.



**Figure: 2.1 Molecular Mechanisms of Hepatocellular Carcinoma at the cirrhotic stage (23)**

## **2.5 Staging and Diagnosis of Hepatocellular Carcinoma**

The method developed by Okuda et al is the most commonly used staging system presently. This system evaluates the albumin levels, size of the tumors and concentration of bilirubin of patients. However, it is ineffective at early stages of the disease. Several prognostic staging systems are currently in clinical use like Barcelona Clinic Liver Cancer, Tumor node metastasis which although advanced does not consider underlying Cirrhosis. Although they all differ on the way of evaluation, they are useful in better understanding of diagnosis.

HCC Tumor is categorized by AJCC, into various stages based on the clinical status of the patient, the size of the tumor, the number of lesions. Stage I and II with the tumor showing some Invasiveness in the blood vessels and forming single to multiple tumors. In stage IIIa, multiple tumors are formed which tend to increase in size and these tumors extend to the hepatic vein by stage IIIb and reach the visceral peritoneum by Stage IIIc. In stage IVa and IV b leads to initially regional to distant metastasis (36) (37).

Diagnosis of HCC involves differential Diagnosis of well differentiated HCCs from lesions and poorly differentiated HCCs from malignancies similar to HCC outside liver. Usually well differentiated HCC's from dysplastic nodules and poorly differentiated HCC's from metastatic tumors, chlorangiosarcoma (38). Diagnosis is mostly possible only when symptoms like weight loss or sometimes much more severe like liver failure are seen and usually suggesting presence of tumored liver (39). Some of the common methods of diagnosis include imaging like CT scan, MRI. The noninvasive nature, fewer costs make imaging more preferable though they are late in detecting only until large



tumors are formed (40). The confirmation of the pathology of HCC play a role in diagnosis and biopsy is a method of doing it. However, it also chances of spreading the disease.

## **2.6 Current Treatment options**

Surgical resection is the preferred method for very early HCC. It is usually indicated in patients without cirrhosis and portal hypertension. Patients with preserved liver function are resected. The only problem it faces is recurrence. However in combination with adjuvant therapy it can be controlled. Liver transplantation is another method for treatment of early HCC and is suitable for patients without proper functioning of liver and any extra hepatic spread or vascular invasion and nodules of size less than 5cm. However, the demand of livers and waiting time is a problem. If the waiting time is greater than 12 months 25% more chances of tumor being spread. Ablation is a method with similar recurrence chances and survival rates when compared to Surgical Resection. It is effective when nodules are below 2cm (41). Percutaneous ethanol injection has been the most successful technique of ablation with little adverse effects. It is time taking and ineffective in tumors larger than 3cm. RFA solves most of these issues of the PEI with greater side effects (39). Some other common ablation techniques are microwave and cryoablation. For patients, with Intermediate HCC and multi nodular tumors without any invasion and extra hepatic spread, Transcatheter Arterial Chemo embolization and Trans-arterial embolization with a radioactive isotope Y90 was used. For patients with Advanced HCC, systemic therapy with Sorafenib, a multi kinase inhibitor is mostly used and few others like Sunitinib, brivonib and being investigated (42) (43).

## **2.7 Current Trends in Research of Biomarkers for Hepatocellular Carcinoma**

HCC is usually asymptomatic and biomarkers therefore act as measurement tools for diagnosis of the disease, its progression and help to target a proper therapeutic treatment. Different types of Biomarkers are presently used worldwide. Alpha-Feto protein is the most commonly used biomarker presently and can be called as a gold standard, it is pretty much useful in patients with known risk factors as its increase is directly in correspondence with the development of HCC (44) (45). However it does not correlate much to the prognostic factors of HCC.

Different types of Immuno Histochemistry markers are useful as diagnostic markers as they use immunochemical staining to distinguishing well differentiated HCC. Some of them are HSP70, Glypican 3. These usually appear and reappear during tumor genesis and usually used in combinations (46). Some of the IHC markers used in prognosis are Ki67, Survivin and E-Cadherin. Enzymes and Iso-enzymes used as biomarkers like Des-Gamma-Carboxy Prothrombin which when used with AFP increases the sensitivity, Gamma Glutamyl Tranferase also is used in combinations with AFP and DCP increases the sensitivity (47). Several growth factors like fibroblast growth factor, epidermal growth factor receptor family and hepatocyte Growth Factor are used. Hepatocyte Growth factor plays an active role as biomarker in prognosis and recurrence. It plays a role in HCC through the panocrine system involving the receptor c-met. Circulating Nucleic acids mainly mRNA, like GGT mRNA, IGF-2 mRNA are analyzed in various pathological and physiological conditions. Micro RNAs provide new insights into HCC carcinogenesis and therefore used in prognosis and diagnosis of the disease.

Depending upon the etiology of the HCC and the molecular abnormalities make it pretty difficult to find a biomarker with good specificity and sensitivity. These are the currently used markers based on the different molecular pathways of HCC and these show great amount of significance and on further research can be used to improve the prognostic values and assist in determining the proper mode of therapy.

## **CHAPTER III**

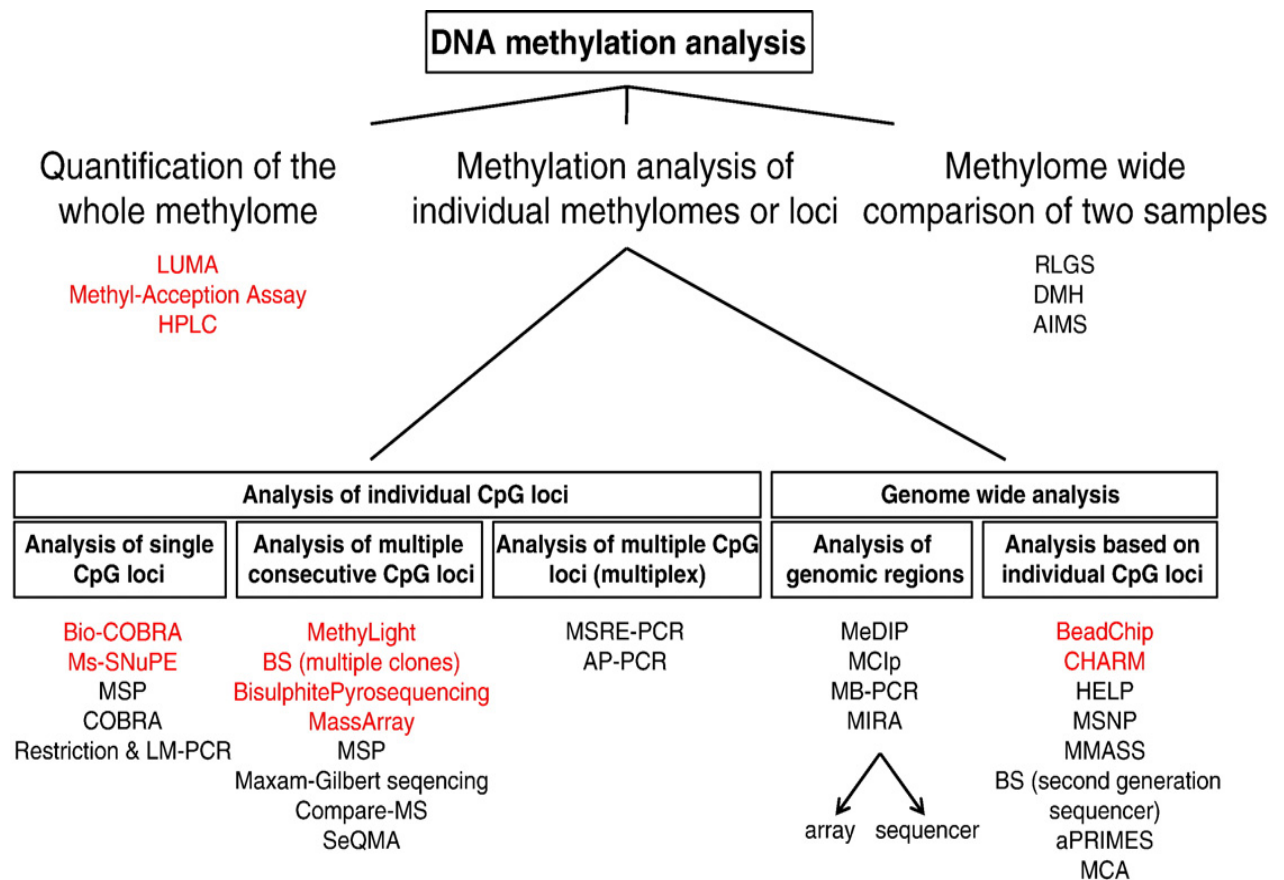
### **DNA METHYLATION ANALYSIS TECHNIQUES**

DNA methylation analysis techniques can be categorized based on chemical modification of molecules, the precipitation of methylated DNA by proteins which interact with the methylated cytosines (48). It is important to choose a protocol on the basis of several factors like sensitivity, cost, specificity and the desired results. In the precipitation techniques, complexes are formed between the proteins and the methylated DNA. These proteins bind and enrich the methylated DNA. Several protocols use different proteins, as in MIRA protocol, a complex of MBDs with glutathione –S-transferase isolates the methylated DNA (49). MBD2 and MeCP2 (methyl binding domain of MBD2 are the most commonly used in isolating methylated DNA (50). MBDs are not specific and are affected by the methylation density of DNA (51). Lack of Specificity and sensitivity limits the precipitation techniques usage.

DNA methylation Analysis techniques using methylation sensitive restriction enzymes which are sensitive to the cytosine methylation in CpG can be used (52). This method involves the division of DNA into two samples and then digests one with enzyme sensitive to the DNA and one without sensitive to DNA methylation and later the missing cuts of enzymes and methylation are compared (48). However, the incomplete digestion,

buffer conditions, over dependence on the sequence of Restriction site and the distribution limits its sensitivity.

Microarrays techniques are generally used in combination with few analysis techniques. High resolution arrays like oligonucleotide arrays have been used in analysis (53). Some of the arrays used are SNP's (54). They cannot quantify the methylation level accurately and therefore acts as a drawback. Methylation Bead chip technology is the currently used microarray technique and it can effectively overcome the drawbacks (55). The techniques which focus on the alterations of DNA depend mainly on the conversion of the methylated DNA by the treatment with sodium bisulfite. The figure 3.1 gives a small description of methods of the DNA Methylation Analysis techniques presently used.



**Figure 3.1: DNA Methylation Analysis Techniques**

### 3.1 Methylation-Specific PCR (MSP)

MSP was one of the early changes which involved Bisulfite Conversion. It was introduced by Herman and colleagues (56). It is a standard PCR reaction used to analyze the methylation status of CpG islands. In this reaction, distinct methylation primer sets for the sequence of interest are used like, the unmethylated primers specific for the unmethylated DNA whereas the methylated primer specific for methylated DNA. The unmethylated cytosines are converted to thymines and methylated cytosines are unconverted and therefore the primers designed should include CpGs of interest.

When compared to other DNA methylation techniques MSP exhibits several advantages based on the simplicity of the procedure, time taken for and lack of need of any specific equipment. It is also less expensive and the presence of larger number of the CpG sites in the CpG islands. These factors aid the use of MSP in larger number of samples with a better sensitivity. It is also used in the prevention of unwanted non tumor cells in the sample, if a uniformly opposite pattern is observed by the tumor cells thereby increasing the detection (57). However it is difficult to obtain good capability as it is a very laborious process.

It depends on various critical factors like Bisulfite Conversion, designing of the primer and PCR.

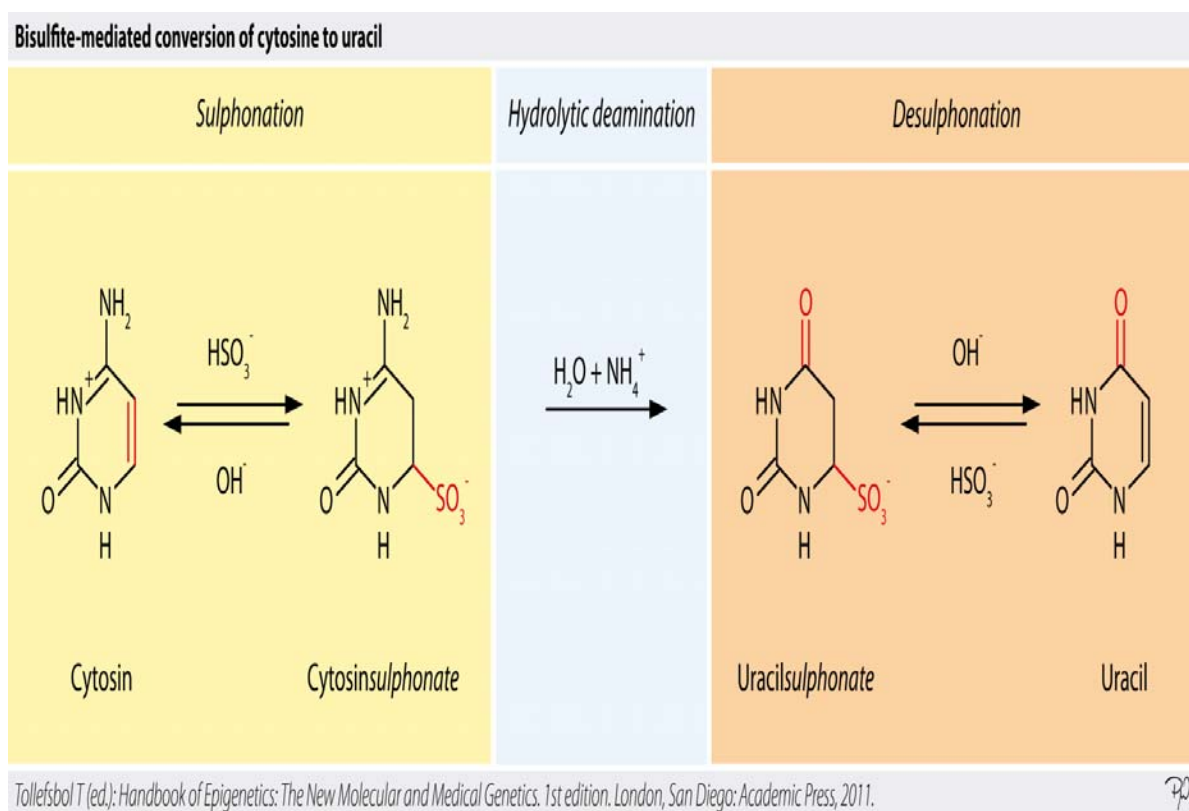
### **3.1.1 Bisulfite conversion**

The existence of 5-methyl cytosine in the DNA is a very important area of study in the present genomic era. One of the most important Analytical method to determine the sites of the 5-Mc in the genome is the bisulfite modification of the genome (58). To understand the role of DNA methylation in various disease states it is very important to quantify and detect the 5-Mc and bisulfite modification is considered as a very efficient method and a gold standard in doing so (59).

#### **Principle of Bisulfite Conversion**

It is based on the selective reactivity of sodium bisulfite with cytosines and methylated cytosines. The chemistry of cytosine deamination involves three steps namely, Sulfonation which involves addition of bisulfite to the 5, 6 bond of the cytosine in the

presence of acidic pH, hydrolytic deamination which is an irreversible conversion of cytosine bisulfite derivative to Uracil bisulfite derivative aided by a free radical scavengers to prevent any oxidization. This is followed by the alkali desulfonation of the Uracil bisulfite derivative to Uracil in the presence of an alkali to decrease the pH and aid in desulfonation (60). Bisulfite treatment deaminates cytosine to Uracil and leave 5-methylcytosine unchanged. One of the major advantage of this method is the integrity of the DNA is maintained and thus enable various techniques for amplifying or analyzing DNA to be performed on the bisulfite modified DNA, thereby can startup with low Concentrations of DNA (48).



**Figure 3.2: Bisulfite Mediated Conversion of Cytosine to Uracil (61)**



The efficiency of the bisulfite conversion is dependent on the following errors like (62), failed conversion when unmethylated cytosine do not undergo deamination and as a result it appears methylated and thus gives an increased estimation of methylation and often it can be corrected by increasing the time of bisulfite treatment or by increasing the number of denaturation steps (63), by doing so can also prevent the renaturation of sample DNA. Inappropriate conversion is another important problem which occurs when methylated cytosine undergo deamination yielding thymine and they like Uracil pair up with adenine during PCR and are misinterpreted as unmethylated leading to underestimation of the methylation density (64). Depurination during the time of conversion is another notable problem by failing of enzymatic reactions when using negligible amounts of DNA (65). Degradation of template DNA is a very common side effect seen and it affects the detection limit of method but how much of DNA is lost during the reaction is not known. Usually, at a temperature of 95 degree Celsius DNA degrades rapidly and thus higher temperatures are preferred only if sufficient amount of DNA is available and it is always advisable to keep the incubation time as low as possible since it is evident that the longer the incubation time greater the degradation (64). However, DNA methylation Analysis on the basis of bisulfite modification are performed on a larger spectrum and many methods of amplification enable a bisulfite converted DNA concentration of 50ng sufficient and thus enabled the design of various techniques based on bisulfite conversion.

### **3.1.2 Primer Design**

The primer design is a very important parameter and is essential in determining the methylation status of a gene promoter. Some of the important parameters to be taken care

while designing a primer are, they should be at a length of 23-24 bp to achieve desired gene specific annealing (66). Exact Annealing temperature of the primer has to be determined, because low stringency leads to mismatching and MSP depends on specific annealing to the DNA. Amplification of the unconverted CpG dinucleotide which is unmethylated may show greater methylation levels. Therefore, the primers should be designed to be specific to Bisulfite modified DNA and also contain few non CpG cytosines in the original template (48).

### **3.2 Applications of MSP**

Various methods of MSP have been developed to increase the sensitivity of detection of MSP for the methylated DNA. Nested MSP is on such method which is more sensitive and allows detection of very small quantities of DNA. However, problems like renaturation, incomplete bisulfite conversion and lack of accurate information about the single strand are seen (67) (68). Quantitative MSP is useful to quantify the number of methylated alleles. It is mainly advantageous as it does not involve steps like gel electrophoresis, enabling it's us as an important tool in fast screening (69).

It is very difficult to find out the exact timing of the methylation changes by MSP. To prevent these types of issues ISH have been used. It was used in combination with MSP and aid in the detection of methylated DNA on tissue slides (70). It is used to examining the progress of tumor, CpG island methylation and gene expression. HPLC is also used and various forms of HPLC like Denaturing HPLC are used in combination with MSP and they detect methylation patterns based on the denaturation temperatures (66). Ion pair-Reverse phase HPLC was also used to distinguish between methylated and

unmethylated with the help of deoxynucleotides (71). Denaturing Gradient Gel electrophoresis is a method based on the thermal stability useful to detect the overall methylation differences and it is the first study to differentiate between methylated, hemi methylated and unmethylated sites (72) (73). These are some of the methods which use MSP as a combination and try to increase the sensitivity of the MSP and aid in better detection between methylated and unmethylated DNA.

## **CHAPTER IV**

### **BIOMARKERS**

A biomarker can be defined as, an indicator used to measure the normal biological function, pathogenicity and response to drug treatment (74). A biomarker can either be secreted by the malignancy itself or as a physiological response to the presence of the cancer. A clinical end point usually determines the efficacy and the biomarkers are mainly used to form an intermediate endpoint and are thus used to monitor earlier stages of carcinogenesis (75). Biomarkers are distinguished mainly into two types, Biomarkers which are used to determine the successful dosage levels required and those which are used to determine the progress of a disease (76). Biomarkers in cancers have multidimensional role (77), they serve in screening studies and diagnosis. Biomarkers used in screening studies usually check the existence of any premalignant conditions or the exposure and in diagnostic role, they are mainly used to diagnosis the presence or absence of cancer in a certain individual. They also constitute a role in the prevention trials of cancer and its treatment as they focus on various effects, like the side effects induced and they further focus on the toxicities rather than the desired result.

The primary defect in Cancer is in the genomic DNA and alterations of DNA like translocation, mutations, aberrant DNA methylation play a major role in carcinogenesis and the biomarkers which can bring about these DNA alterations are used as potential tumor biomarkers. The use of DNA as a tumor marker has several advantages. When in comparison with proteins they can be amplified and therefore useful in the detection of minute quantities of the samples. High stability when compared to m-RNA and the proteins which aids its survival in adverse conditions for longer periods (78).

#### **4.1 DNA Methylation as a bio-marker**

The aberrant methylation of the promoter regions of genes is seen both in early as well as advanced cancers and when DNA with these aberrantly methylated genes is released into the blood serves as an indicator for the earlier detection of the cancer. This ability to detect cancer in its earlier phases makes DNA Methylation an important marker over other markers which cannot detect cancer in its earlier phases of tumorigenesis (79). The involvement of DNA methylation in both onset as well as progression of different cancers enables it to serve a dual role both as a diagnostic and a prognostic marker of disease. The main advantage of DNA methylation over mutations which can arise at any point in the gene is its site specificity thus increasing its sensitivity of detection.

##### **4.1.1 DNA methylation as a Diagnostic Marker**

Early diagnosis is very critical for the successful treatment of cancer. The traditional methods of diagnosis invasive as well as noninvasive like cytology, histopathology and immunohistochemistry are useful. The detection of cancer with minimal invasive procedure is always preferred. The changes in the marker should be specific so as to

differentiate the level of malignancy and assess the premalignant conditions, thereby diagnosis the risk level of the progression of the malignancy.

The sensitivity and the specificity of the DNA methylation marker depends on many factors in cancer diagnosis namely the type of body fluid used, type of cancer studied and techniques involved (6). DNA methylation biomarkers can show few premalignant conditions which are not progressed to a level of detection detectable by other methods of detection by quantitative analysis of the levels before its progression to a fatal limit of malignancy. Some of the available diagnosis methods might show false results leading to further analysis and therefore costing time, money as well as psychological stress. This can be avoided by using DNA methylation markers in combination with other tests as a panel and therefore increasing the sensitivity and specificity of the screening procedures.

#### **4.1.2 DNA Methylation as a prognostic and predictive bio-marker**

The genes which undergo methylation at any stage of the cancer progression act as potential prognostic markers whereas predictive biomarkers are the markers based upon the therapeutic results (80). For determining the best therapeutic mode of treatment, patient's response to the administered chemotherapeutic agent and survival, thereby allowing monitoring of various changes accordingly, these markers are utilized. Usually prognosis is carried on in absence of the adjuvant therapy.

Predictive markers are usually evaluated when measurable amount of disease is usually seen whereas prognostic effect is usually evaluated as a control without any systemic treatment. Prognostic factors are mainly utilized to differentiate patients based on the aggressive of the disease thereby treating them accordingly by avoiding the unnecessary

side effects. However it is still unclear in many reports whether the predictive or the prognostic impact has been observed.

#### **4.2 p16 as a biomarker**

p16INK4 is a tumor suppressor gene which negatively regulates the cell cycle by binding to the cyclin-dependent kinases and inhibiting them. The p16INK4 is located on chromosome 9p21 and is one of the most altered genes in human cancers (81). The entry and progression of cells through different phases of cell cycle with the aid of cyclins which form complexes with group of constitutively expressed proteins called cyclin dependent kinases (CDKS) and P16 is a cyclin dependent kinase Inhibitor of CDK4 and CDK6 which participation in the cyclin dependent phosphorylation of the retinoblastoma protein (pRB) regulatory pathway.

The exact mechanism of action of p16 as TSG is not clear. However, p16 is usually inactivated in three ways homozygous deletion, promoter methylation and point mutations in tumors which retain wild type Rb and ectopic expression of these cells result in G1 arrest causing its frequent inactivation and homozygous deletion aids not only in development but also in prognosis of several tumors (82).

#### **4.3 SLIT-2 as a biomarker**

Slit-2 is a novel tumor suppressor gene and belongs to the SLITs family which comprises of ECM secreted and membrane associated glycoproteins. SLIT2 and its another two members SLIT1 and SLIT-3 are the ligands for the ROBO-gene family, which are the receptors for interactions between axons and growth cones during neuronal development.

Expression of SLIT-2 in non-neuronal tissues apart from neuronal ones shows its role outside its neural importance.

SLIT-2 gene maps on the chromosome 4p15.2 and it consists of a argin-laminin-perlecan-slit conserved space motif, four leucine rich repeats, nine epidermal growth factors repeats and a cysteine knot (83). The interaction of SLIT-2 with ROBO-1 is through its leucine rich repeats. SLIT-2 has been found to have a profound role in tumorigenesis, invasion, metastasis and prognosis of various cancers (84).

SLIT-2 is considered as a TSG because of its frequent inactivation in a wide variety of cancers due to hyper methylation of its promoter region. SLIT-2 exhibits its tumor suppressive effect by a modulating the beta –catenin-wnt signaling pathway. Beta-catenin is considered to be an oncogene and its deregulation or activation of mutations can lead to carcinogenesis (85). The activation of this pathway plays an important role in human hepatocellular carcinoma. Wnt/beta-catenin pathway plays an essential role in all phases of liver development like development, regeneration and maturation and is essential for the metabolic function of liver (86).



## CHAPTER V

### MATERIALS AND METHODS

#### 5.1 Bisulfite Conversion

Prior to the sodium bisulfite conversion, the DNA concentration was quantified by RT-PCR amplification. Standard methylated and unmethylated genomic DNA was purchased for establishing the standard curve. Different concentrations of the standard DNA are obtained by dilution and then amplified with the ACTB primer ( $\beta$ -actin) and sample DNA concentration was calculated using the standard calibration curve. In comparison with these results the final genomic DNA concentrations of all the samples were normalized.

ACTB primer: F: 5'GGCGGCACCACCATGTACCCT 3'

R: 5'AGGGGCCGGACTCGTCATACT 3'

Sodium bisulfite Reaction was performed on normal and cancerous DNA samples as well as standard methylated and unmethylated samples using the EZ DNA METHYLATION Gold Kit (Zymo Research, Irvine, CA)

### 5.1.1 Reagent preparation

#### 5.1.1.1 Preparation of CT-conversion Reagent:

The CT conversion supplied is a solid mixture and 900µL water, 300 µl M-Dilution buffer and 50µl M-dissolving buffer to a tube of CT conversion Reagent. It is thoroughly mixed with frequent vortexing for 10 minutes. It can be stored at -20°C.

It can also be prepared manually,

**Table 5.1 CT-conversion Reagent**

<b>Reagent</b>	<b>Composition</b>	<b>Quantity 96 samples</b>
Sodium metabisulphite	Na <sub>2</sub> S <sub>2</sub> O <sub>5</sub>	5.8g
M-Dilution buffer	2MNaOH	2880µL
M-Dissolving buffer	50%dimethyl formamide	480µl
water	H <sub>2</sub> O	1200µl

Light should be avoided as it is very sensitive and best if used immediately after preparation.

#### 5.1.1.2 Preparation of M-wash buffer

24ml of 100% ethanol to the 6ml M-wash buffer or 96ml of 100% ethanol to the 24ml M-wash buffer concentrate should be added before use.

### 5.1.2 Protocol for bisulfite Conversion

1. 130  $\mu$ l of the CT conversion reagent prepared is added to 20 $\mu$ l of the DNA sample in a PCR Tube. The amount of the input DNA can be from 500pg-2 $\mu$ g. However for optimum concentrations 200-500ng is preferred.
2. If the DNA sample is less than 20 $\mu$ l, the difference can be made up by water.
3. 2 or 3  $\mu$ l of Salmon DNA is added to the sample.
4. Place the sample tube in the thermo cycler and reaction proceeds in following steps
  - 98°C for 10min
  - 64°C for 150min
  - 4°C storage up to 20 hours
5. The whole process for purification consists of binding of sample, desulfonation, binding buffer wash, ethanol wash and elution.
6. 600 $\mu$ l of the M-binding buffer is added to the zymo spin IC column and column is placed into a collection tube of capacity 800 $\mu$ l.
7. The sample is added to the column and is inverted several times. It is centrifuged at full speed for 30s and flow through is discarded.
8. 200 $\mu$ l of the M-desulphonation buffer is added to column and left at room temperature for 15-20min. After the incubation, it is centrifuged for 30s.
9. 200 $\mu$ L of wash buffer is added twice and centrifuge after each for 30s.
10. At the end the column is placed into a 1.5ml micro centrifuge tube and M-elution buffer (10Mm of Tris buffer) is added. It is centrifuged for 30s and DNA is eluted

DNA can be used immediately or stored at -20°C for further use. For longer time it should be stored below -70°C

## 5.2 DNA Methylation Analysis

MSP amplifications of 2genes p16 and SLIT-2 were performed on Real-Time PCR instrument .The primer sequences are mentioned in the table below,

**Table 5.2 The MSP primer sequences of genes**

Gene	MSP primer 5'-3'	Annealing Temperature(°C)
p16	F:TTATTAGAGGGTGGGGCGGATCGC R:CCACCTAAATCGACCTCCGACCG	66
SLIT-2	F:GGGAGGCGGATTGTTTAG R:CATAACGCGCGAAAATACAC	52

### 5.2.1 Preparation of primer

The initial concentration of the primer is 100µg.It is available in dry form. It is re-suspended by dissolving in the appropriate amount of water. It is then diluted to 5µmol/L

### 5.2.2 HRM master mix composition

DNA polymerases, SYBR green dye, optimized concentration of Q-solution NTPs and MgCl<sub>2</sub>. The reaction mixture included 1µl of bisulfite –modified DNA, 5µl of HRM master mix 2x, 1µL primer solution at 5µmol/L and elution buffer to make up to 10µl.

The following PCR program was applied:

95°C denaturing for 10min

41 cycles of next three steps

95°C for 45s.

Annealing temperature for 30s

Extension at 72°C for 45s

### **5.3 Standard Curve Analysis**

Standard Curve was prepared for the standard DNA sample which is bisulfite converted. The standard Curve was plotted at different concentrations and the concentrations were diluted with elution buffer. The concentrations used were 28ng/μl, 14ng/μl, 7ng/μl and 3.5ng /μl. The Standard curve of ACTB-B (reference gene) with the CT plotted against the log of the starting quantity of template for each dilution. The same was done for p16 and SLIT-2. The amplification efficiency and the  $R^2$  value were calculated.

### **5.4 Sensitivity and Specificity of the primer**

The specificity of the MSP was determined by detecting both the methylated and unmethylated DNA modified with Bisulfite Conversion. The sensitivity of the MSP was determined by mixing various patterns of Methylated and Unmethylated Standard DNA like 1% methylated, 10% methylated, 100% methylated and 100% unmethylated. These percentages of methylated and unmethylated standard DNA were prepared by using different concentrations of methylated and Unmethylated DNA.

## CHAPTER VI

### RESULTS AND DISCUSSION

#### 6.1 Standard curve analysis

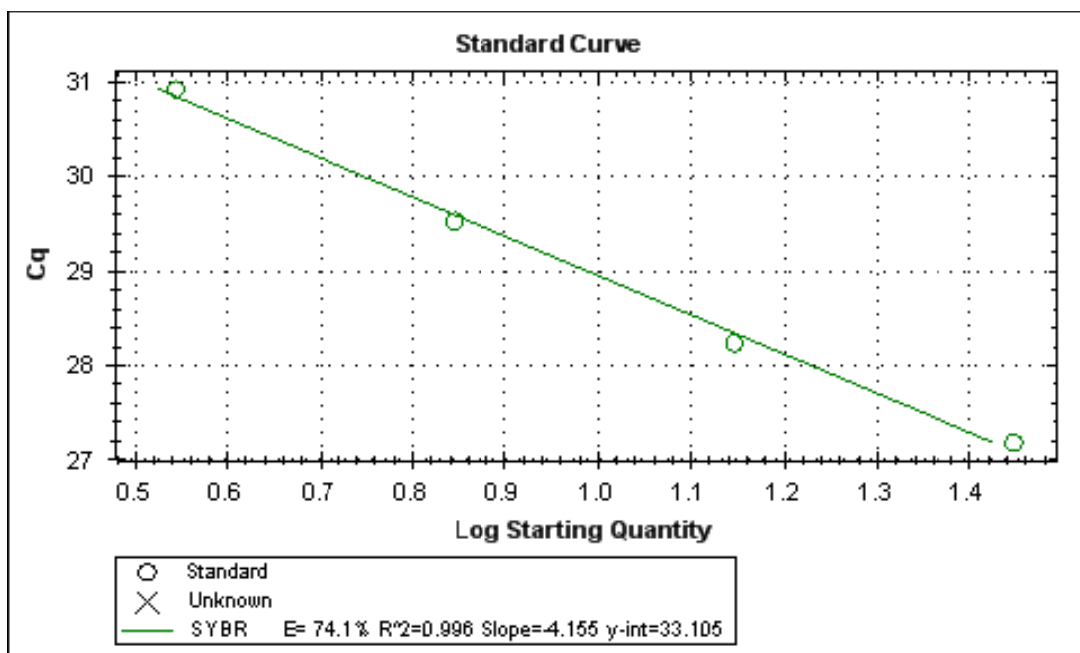
To determine the efficiency of the given gene the standard curves were plotted at different concentrations and  $R^2$  value was calculated.

#### ACTB-B

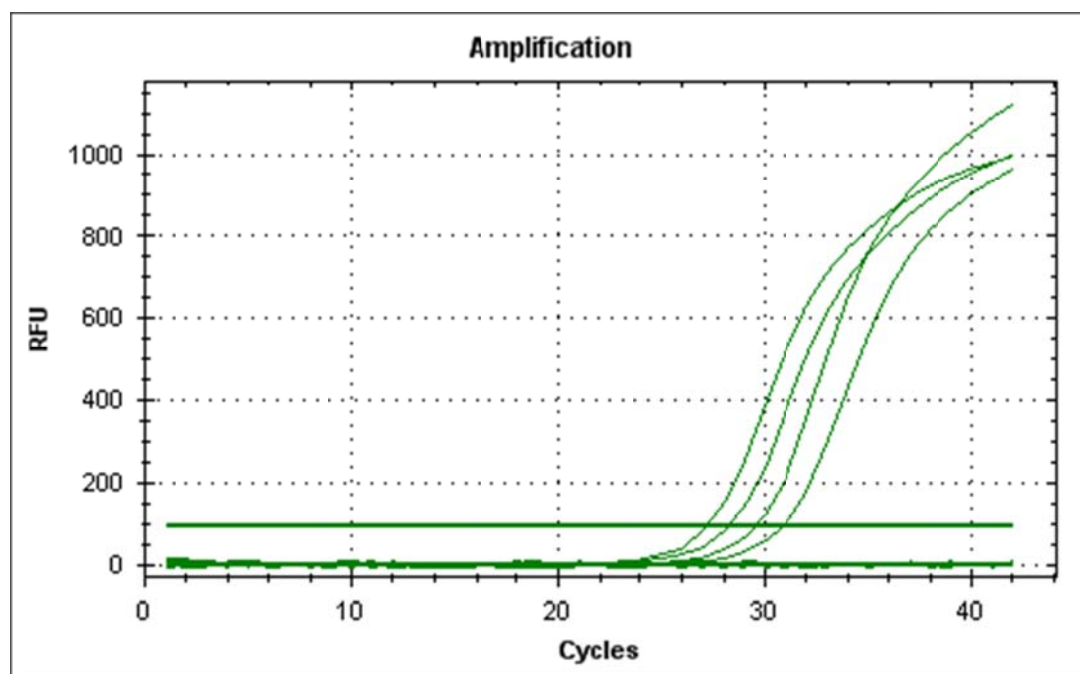
**Table: 6.1      Standard Curve Analysis for the reference ACTB-B**

<b>Conc.(ng/μl)</b>	<b>Ct</b>	<b>Melting temp(°C)</b>
28	27.18	75.60
14	28.24	75.40
7	29.53	75.40
3.5	30.92	75.60

**$R^2=0.996$**



**Figure 6.1: Standard curve of ACTB-B (reference gene) with the CT plotted against the log of the starting quantity of template for each dilution**



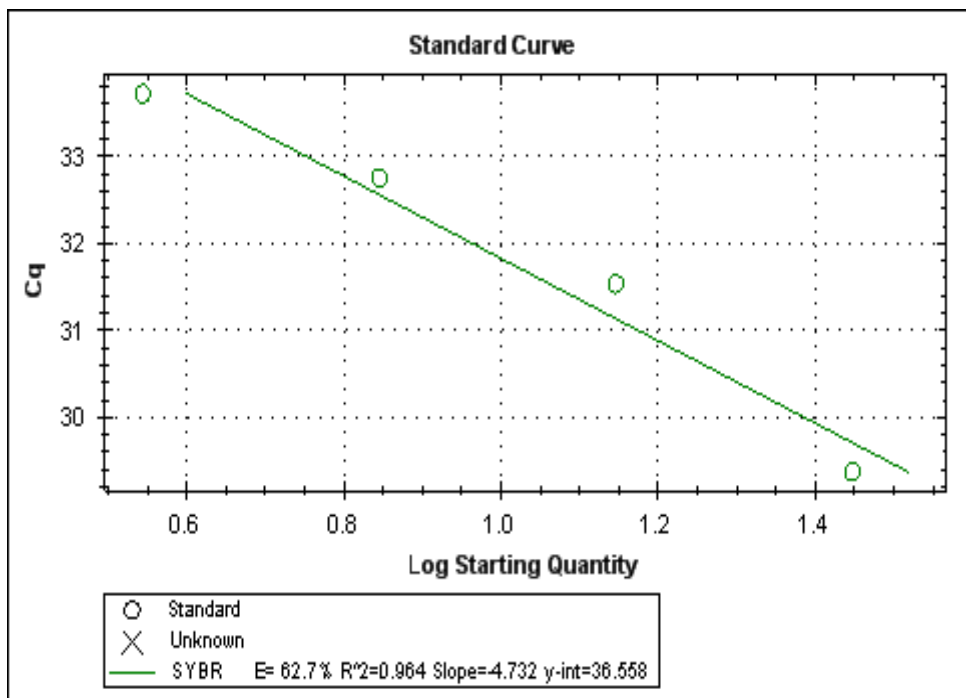
**Figure 6.2: Amplification Curve for the ACTB-B Standards**

P16

**Table: 6.2     Standard Curve Analysis for the gene p16**

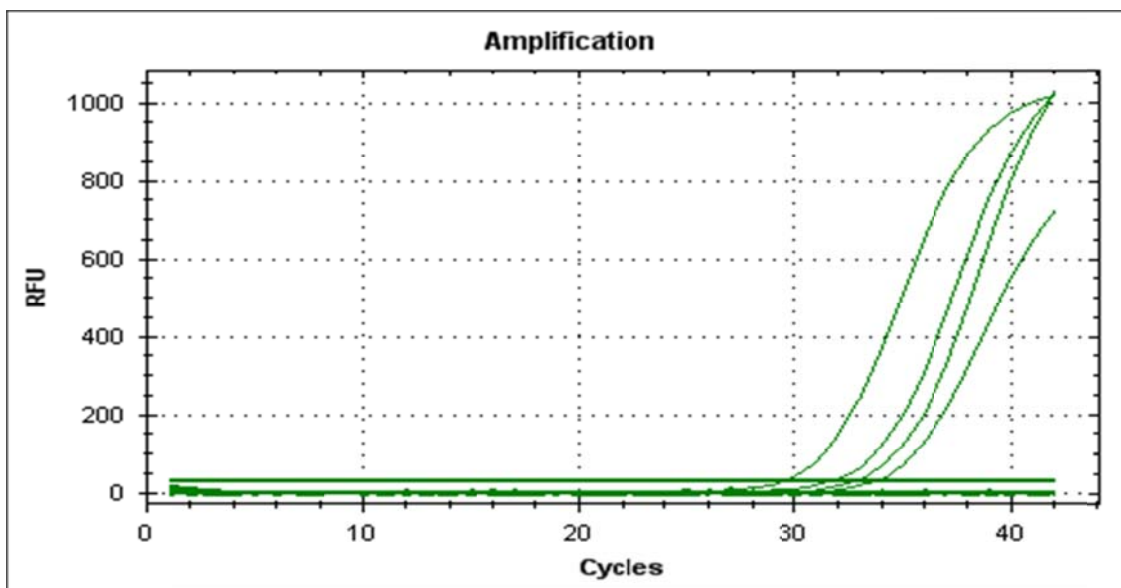
conc (ng/μl)	Ct	Melting Temp(°C)
28	29.38	88.00
14	31.53	88.00
7	32.76	88.00
3.5	33.72	88.00

$R^2=0.964$



**Figure 6.3:     The standard Curve of p16 with the CT plotted against the log of the starting quantity of template for each dilution.**





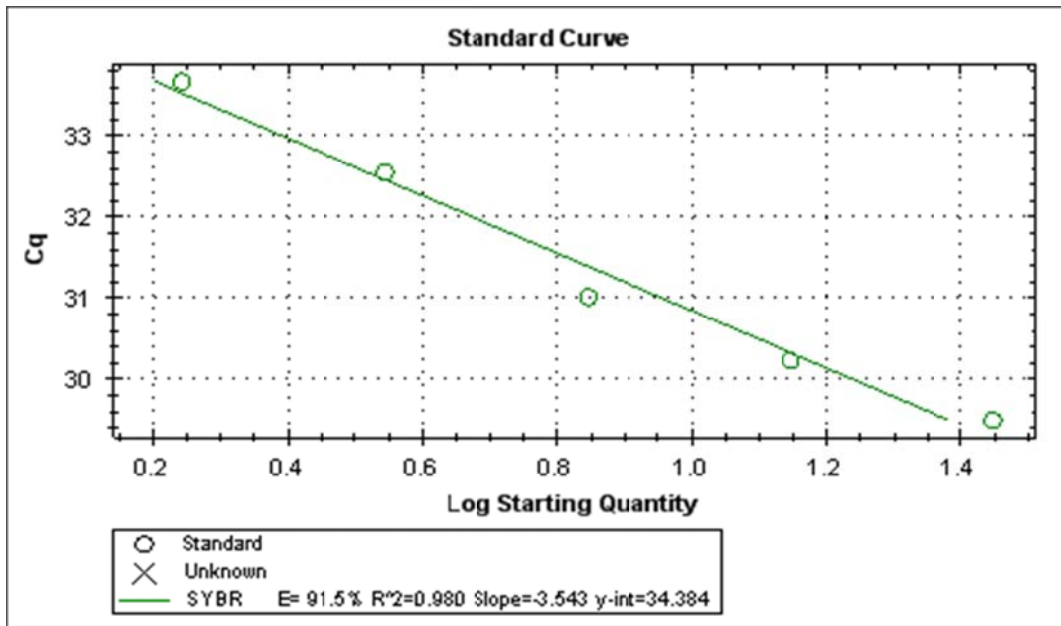
**Figure 6.4: Amplification Curve for the p16 Standards**

## SLIT-2

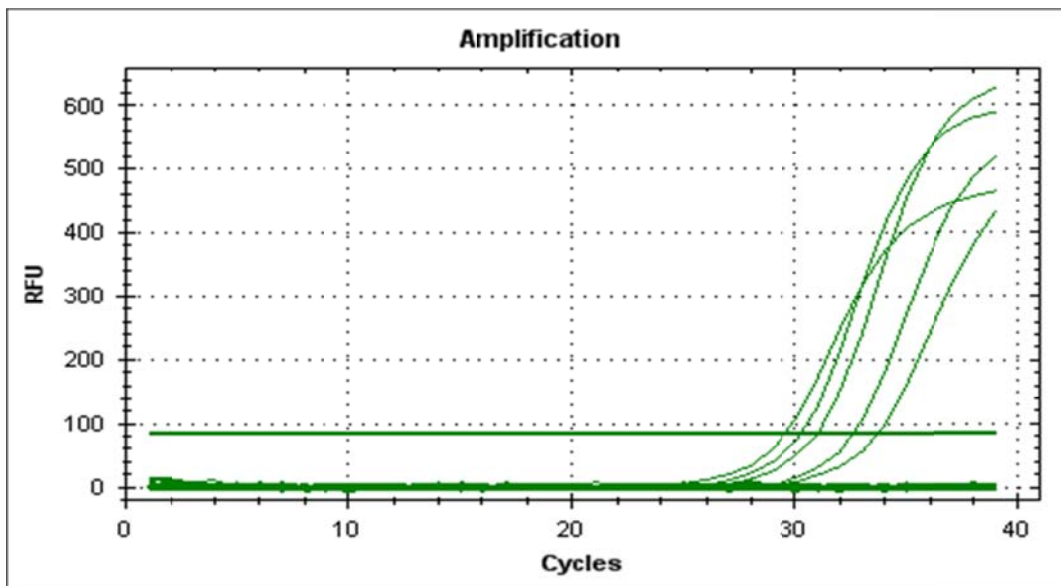
**Table: 6.3. Standard Curve Analysis for the gene SLIT-2**

conc( ng/μl)	Ct	Melting Temp(°C)
28	29.49	84.20
14	30.23	84.40
7	31.01	83.80
3.5	32.55	84.20
1.75	33.67	84.40

**$R^2=0.98$**



**Figure 6.5:** the standard Curve of SLIT-2 Standard curve with the CT plotted against the log of the starting quantity of template for each dilution.



**Figure 6.6:** Amplification Curve for the SLIT-2 Standards

## 6.2 Gene specific methylation analysis

During this epigenetic study of HCC aberrant promoter methylation of p16 and SLIT-2 was reported in previous studies. These genes were selected for methylation screening. Four pairs of patient samples with considerable amount of DNA were selected and used in this methylation profiling.

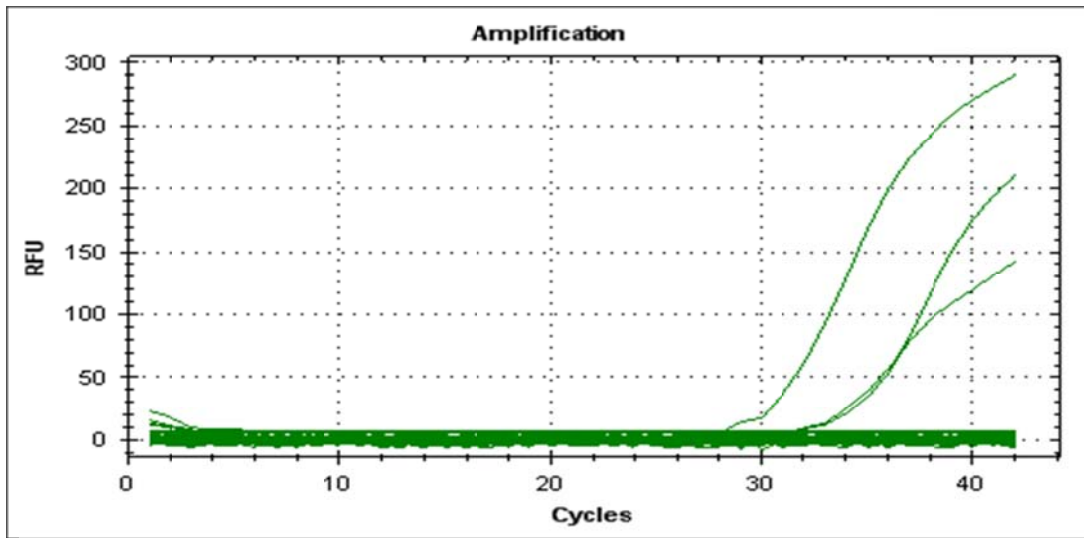
The methylation rates for the two genes were as followed,

**Table 6.4: The methylation profiles of the two genes**

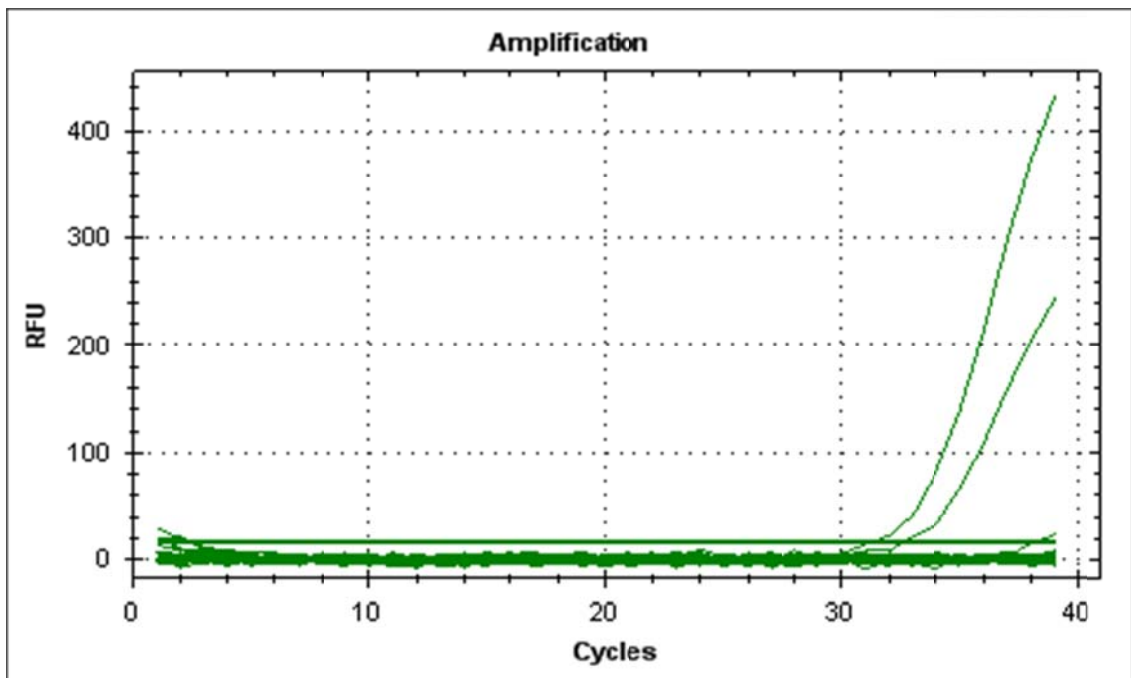
Gene	Cancerous liver tissue		Non-Cancerous liver tissue	
	No of samples methylated	Methylation rate	No of samples methylated	Methylation rate
P16	0(4)	0	0(4)	0(4)
SLIT-2	2(4)	50%	3(4)	75%

## 6.3 Specificity and Sensitivity of the genes

The specificity of the MSP methods on the two genes was evaluated using standard unmethylated and methylated DNA. This highly specific test on the reference gene and SLIT-2 was illustrated in the figure 6.7 and 6.8 below by the PCR amplification curve for each respectively. The selected primers and the optimized PCR conditions ensured that only methylated DNA after bisulfite conversion could be amplified for the two genes.



**Figure 6.7: Amplification Results of standard methylated DNA, 10% methylated DNA, standard unmethylated DNA with ACTB-B**



**Figure 6.8: Amplification Results of standard methylated DNA, 10% methylated DNA, standard unmethylated DNA with SLIT-2**

## 6.4 DISCUSSION

Epigenetic Analysis of DNA Methylation plays a very important role in tumorigenesis and aid in the discovery of an efficient biomarker for the diagnosis, prognosis of Hepatocellular Carcinoma. In this study, we investigate the methylation status of SLIT-2 and p16 in cancerous and noncancerous tissue samples. With each gene involved in different molecular pathways, p16 involved in the cell cycle Regulatory pathway and SLIT-2 involved in the beta –catenin-wnt signaling pathway. It is very important to understand their functions in Hepatocellular Carcinoma to predict the conditions for earlier diagnosis.

Standard Curve Analysis was performed on various concentrations of genomic Standard DNA and graphs were plotted and  $R^2$  values were determined. Standard Curve Analysis was performed to determine the Amplification Efficiency of the PCR and they were determined for the reference gene, p16 and SLIT-2. The Standard Curve was plotted for log of starting quantity and Ct values. The correlation coefficient ( $R^2$ ) values determine the accuracy of the reaction and a good agreement between concentration and fluorescence.  $R^2$  values usually range from 0-1 with 1 being the best. The value of the reference gene ACTB-B was found to be 0.996, p16 was 0.964 and SLIT-2 was 0.980 showing the efficiency of the MSP.

In the present study alterations of the methylation status of p16 and SLIT-2, where sodium bisulfite react with methylated Cytosine's specifically and aid in discrimination of methylated DNA by MSP. Gene specific alterations of promoter methylation status of SLIT-2 and P16 were investigated using bisulfite Conversion and MSP. Different pairs of

cancerous and noncancerous samples were tested and methylation status was examined. Initially all the samples were screened with ACTB-B which is a reference gene and only those samples which yielded positive results were further evaluated. P16 and SLIT-2 methylation status were estimated by calculating the number of methylated samples over the total number of samples. Although hyper methylation of p16 was reported in earlier studies of HCC, the present study yielded negative results for all the pairs of samples. SLIT-2 showed methylation both in cancerous and non-Cancerous samples.

In order to determine the efficiency of a gene as a Biomarker for Hepatocellular Carcinoma different categories have to be considered like specificity, sensitivity. The specificity of each gene was estimated on its ability to distinguish between unmethylated and methylated samples. It was found from the above results both p16 and SLIT-2 were specific enough as they did not give any signal for the Unmethylated Standard DNA and a positive signal was seen for the methylated DNA. The sensitivity of each gene was determined by its ability to determine various methylation percentages and we could see SLIT-2 showed signal with all the methylated percentages up to 1 whereas p16 did not give any signal for 1% methylated DNA.

Though p16 which is one of the successful biomarker for HCC, it yielded negative results probably due to problems with its primer design. SLIT-2 yielded average results, thus making it useful as a biomarker. In conclusion, two genes were investigated for gene specific alterations through promoter methylation using bisulfite conversion and Methylation Specific PCR. In both cancerous and non-cancerous tissues CpG island hyper methylation was found in SLIT-2. In p16 no evident methylation was seen.

## **CHAPTER VII**

### **SUMMARY**

Cancerous and non-cancerous pairs of tissue samples were extracted from the FFPE tissues and samples are bisulfite converted where the positive display of 5-methyl cytosine's in the gene promoter after bisulfite modification based on the selective reactivity of the sodium bisulfite to methylated and unmethylated Cytosine's. The MSP is the method of DNA methylation Analysis used with analysis of methylation status of CpG islands with distinct methylation primer sets for the sequence of interest. p16, a gene involved in the cell cycle Regulatory pathway and SLIT-2 involved in beta –catenin-wnt signaling pathway were evaluated as DNA methylation biomarkers based on amplification of the two genes on Real time PCR using Melting Curve Analysis. The samples were tested initially with the reference gene  $\beta$ -actin and Standard curves have been plotted for the genes of interest and the reference gene and  $R^2$  values were calculated. Gene promoter Methylation Analysis of the genes was done to analyze the methylation rate of both the genes in cancerous and non-cancerous samples. The specificity and sensitivity of each gene was assessed by using different methylation percentage.

Thus, p16 yielded negative results and SLIT-2 moderate results as Biomarkers for HCC. The results from the present study show the role of DNA methylation as an important part of hepato-carcinogenesis and its importance as a Biomarker.



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