BIOMARKER RESEARCH

DNA methylation: potential biomarker in Hepatocellular Carcinoma

Way-Champ Mah[1,2,3] and Caroline GL Lee[1,2,3,4*]

Abstract

Hepatocellular Carcinoma (HCC) is one of the most common cancers in the world and it is often associated with poor prognosis. Liver transplantation and resection are two currently available curative therapies. However, most patients cannot be treated with such therapies due to late diagnosis. This underscores the urgent need to identify potential markers that ensure early diagnosis of HCC. As more evidences are suggesting that epigenetic changes contribute hepatocarcinogenesis, DNA methylation was poised as one promising biomarker. Indeed, genome wide profiling reveals that aberrant methylation is frequent event in HCC. Many studies showed that differentially methylated genes and CpG island methylator phenotype (CIMP) status in HCC were associated with clinicopathological data. Some commonly studied hypermethylated genes include p16, SOCS1, GSTP1 and CDH1. In addition, studies have also revealed that methylation markers could be detected in patient blood samples and associated with poor prognosis of the disease. Undeniably, increasing number of methylation markers are being discovered through high throughput genome wide data in recent years. Proper and systematic validation of these candidate markers in prospective cohort is required so that their actual prognostication and surveillance value could be accurately determined. It is hope that in near future, methylation marker could be translate into clinical use, where patients at risk could be diagnosed early and that the progression of disease could be more correctly assessed.

Keywords: Epigenetics, Methylation, Biomarker, CIMP, Hepatocellular carcinoma, Prognosis, Diagnosis

Introduction

Hepatocellular Carcinoma (HCC) is one of the most frequent cancers in the world and annually, about 600,000 patients died of liver cancer [1]. This disease is often associated with poor prognosis because patients are either diagnosed at very late stage or experienced recurrence after resection [2]. In fact, more than half of HCC patients died within 12 months post diagnosis, and less than 6% of them have an average survival rate of 5 years [3]. Liver transplantation and resection are the only two curative therapies available; however, in order to qualify for such therapies, patients need to be diagnosed early with HCC [4]. Presently, serum alpha-fetoprotein (AFP) concentration and hepatic ultrasonography are used in

HCC surveillance program, where high risk patients are screened for HCC in every six months [5]. As for actual diagnosis, invasive biopsy and expensive imaging tools such as ultrasonography, spiral computed tomography (CT) and magnetic resonance imaging (MRI) are used [4]. AFP measurement is merely used as adjunct diagnostic tool because of its variability in specificity and sensitivity [5,6]. Equally important to note is that apart from AFP level and tumor staging classification such as the Barcelona Clinic Liver Cancer (BCLC) staging system, there is no good prognostic marker that can classify patients and predict survival outcome [7-9]. The large number of HCC associated deaths clearly reflects the shortcomings of current diagnostic and prognostic tools. This underscores the importance of discovering novel and effective biomarkers that can improve overall clinical management of HCC.

With the advance of genomic technologies, plethora of molecular data is now available for translational research. Gene expression signatures and microRNA profiles are

* Correspondence: caroline_lee@nuhs.edu.sg
[1]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore
[2]Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, National Cancer Centre Singapore, Level 6, Lab 5, 11 Hospital Drive, Singapore 169610, Singapore
Full list of author information is available at the end of the article

just some examples of molecular data that were actively explored as potential biomarkers for HCC [10-15]. In this review, we will focus specifically on DNA methylation, another potential biomarker that was shown to be implicated in HCC.

Review

Aberrant DNA methylation in HCC

Studies have identified a few somatic mutations in HCC, for instance, mutations in TP53 [16,17], CTNNB1 [18,19], and AXIN1 [20,21]. However, frequencies of these mutations are inconsistent and rare, some occur only in certain subtypes of tumor [22]. The lack of common genetic marker associated with HCC cases strongly suggests that epigenetic alterations such as aberrant DNA methylation could be the alternative factor contributing towards liver carcinogenesis. DNA methylation occurs when a methyl group is attached to the 5th carbon of cytosine nucleotide and this process is catalyzed by DNA methyltransferases (DNMTs), in which S-adenosyl-methionine (SAM) acts as a methyl donor (Figure 1) [23]. Deregulation of DNA methylation was shown to be associated with many cancers, as was first proposed by Andrew Feinberg and Bert Vogelstein in 1983 [24]. The two most common forms of aberrant methylation are global hypomethylation and site-specific hypermethylation. In HCC, such deregulations are frequently observed as well. Global hypomethylation in liver cancer affects the structural-nuclear function by promoting chromosomal and genomic instability, while regional hypermethylation is often associated with silencing of tumor suppressor genes [25]. Studies have revealed that etiological factors like Hepatitis virus infection may lead to aberrant DNA methylation in cancerous tissues [26,27]. DNA methyltransferases such as DNMT1, DNMT3A and DNMT3B were also shown to be up-regulated in liver cancer [28,29]. Whether increased expression of DNMTs associated with aberrant methylation of genes is still a matter of controversy as the exact mechanism has yet to be elucidated [29,30]. Subsequent sections will summarize

respective studies on aberrant DNA methylation in hepatocarcinogenesis and the full list of studies can be found in Additional file 1: Table S1.

Genome wide studies on methylation profile of HCC

Present technologies allow researchers to profile methylation in a genome wide manner. Two commonly used methods in HCC methylation profiling include hybridization of bisulfite converted DNA on beadarray [31,32] and enrichment of methylated DNA either by enzymatic digestion [33,34] or antibody pull-down [35], followed by promoter array profiling. Even though high throughput sequencing is becoming more available, presently, no study has yet to use this approach to map the methylome of HCC. Table 1 briefly summarizes the strengths and limitations of each profiling method. A few pivotal genome wide methylation studies using these approaches are highlighted below.

In 2008, Gao et al. adopted methylated CpG island amplification microarray (MCAM) method to identify 719 genes that were differentially methylated between tumors and adjacent non-tumors [36]. They used pyrosequencing to validate their observations found by MCAM. Genes such as RASSF1A, CDKN2A and CCNA1 were successfully validated to be highly methylated in cancer tissue compared to adjacent non-tumor and normal liver tissues. In subsequent year, Lu et al. used differential methylation hybridization (DMH) method to locate 38 hypomethylated and 27 hypermethylated regions. Using Methylation specific PCR (MSP) method, they validated the methylation status of KLK10 and OXGR1 in tumors, and found that hypermethylation of KLK10 was associated with Hepatitis C virus (HCV) infection and cirrhosis [37]. Around the same time, studies by Deng et al. and Stefanska et al. used a slightly different method called methylated DNA immunoprecipitation microarray (MeDIP-chip) to locate aberrant methylation in HCC. Deng et al. used MassArray® method to validate hypermethylation of DUSP4, NPR1 and CYP24A1 in HCC, and correlate methylation status
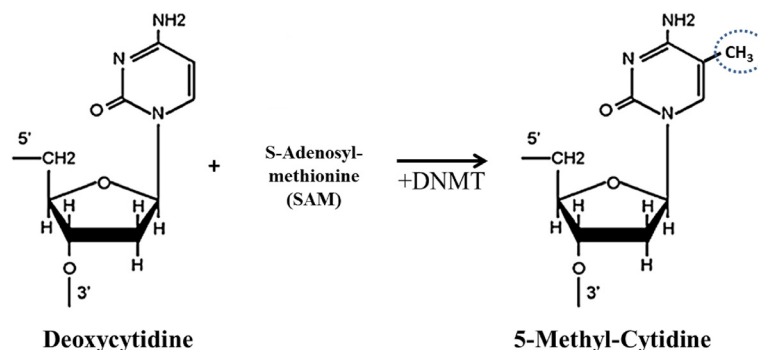

Figure 1 Structures of deoxycytidine and 5-methyl-deoxycytidine. DNA methylation occurs when a methyl group is attached to the 5th carbon of cytosine, where DNMT serves as enzyme and SAM acts as the methyl group donor.

Table 1 Different methods in genome-wide methylation profiling

	Platform	Features	Number of regions analysed per sample	Methylation information on site specific CpG loci	Methylation information on non-CpG loci	Advantages	Disadvantages
Microarray based	Methylated CpG Island Amplification and Microarray (MCAM-chip)	Enzyme-based techniques that rely on restriction enzymes (SmaI and XmaI) followed by profiling on promoter array	~25,000 human promoters (depends on array density)	No	No	Do not require bisulfite conversion, good coverage on region with low CpG density.	Require substantial quantities of input genomic DNA, low sample throughput, do not report methylation status at single nucleotide level, bias may occur due to genomic distribution of CpG loci, limited to mostly promoter regions.
	Differential Methylation Hybridization and Microarray (DMH-chip)	Enzyme-based techniques that rely on restriction enzymes (MseI and BstUI) followed by profiling on promoter array					
	Methylated DNA Immunoprecipitation and Microarray (MeDIP-chip)	Immunoprecipitation of methylated DNA with a monoclonal antibody followed by profiling on promoter array					
Beadarray based	GoldenGate	Bisulfite convertion of DNA followed by microbead based microarray	~1,500 CpG sites	Yes	No	Require minimum input genomic DNA, high sample throughput, provide methylation status at CpG loci, fairly accurate and reproducible.	Bisulfite treatment may not be complete, bisulfite treatment caused DNA degradation, limited to mostly promoter regions.
	Infinium 27K		~27,000 CpG sites				
	Infinium 450K		~450,000 CpG sites				
High throughput sequencing	Bisulfite sequencing	Bisulphite conversion of DNA followed by capture and high throughput sequencing	Whole genome	Yes	Yes	High resolution mapping of methylation status at single nucleotide level, no cross hybridization bias.	Bisulfite treatment may not be complete, bisulfite treatment caused DNA degradation, low sample throughput, expensive, complex bioinformatic analysis.

of these genes with recurrence free survival [38]. Stefanska et al., on the other hand, delineated the profile of promoter hypomethylation in HCC and validated AKR1B10, CENPH, MMP9, MMP12, PAGE4, S100A5, MMP2 and NUPR1 to be hypomethylated in liver cancer using pyrosequencing [39]. Earlier genome wide studies utilised promoter microarray to map out differentially methylated regions. As a result, such arrays could not provide information on site specific CpG dinucleotides that were aberrantly methylated. Additional validation steps such as pyrosequencing and MassArray® were required before one could locate the exact deregulated CpG sites. Nonetheless, as technology of methylation profiling matures over recent years, many studies could now report genome wide methylation status of HCC at single-nucleotide resolution (Table 2). These studies mainly used the beadarray technology developed by Illumina®. As shown in Table 2, the most recent studies by Song et al., Zhang et al. and Shen et al. reported the mapping of more than 485000 CpG sites, the highest throughput so far, in HCC.

Genome wide methylation profiling provides wealth of information for downstream analysis. Using these high throughput data, researchers could efficiently separate tumors from adjacent non-tumors [39-47], cirrhotic liver from HCC [48,49], and cluster the tumors according to their risk factors such as viral infection [38,43,46] and alcohol consumption [42,43]. Novel tumor suppressor genes that were silenced by methylation could also be uncovered through genome wide studies, as shown by studies in [37,50-53]. As reports on genome wide methylation profile of HCC patients using serum DNA begin to emerge [41,42], it is hoped that these high throughput data could accelerate the process of biomarker discovery.

Methylation as prognostic marker
From 2003 to 2013, many studies have published on the prognostic values of DNA methylation in HCC. These studies are summarized in Additional file 1: Table S1. Due to space constraints, within this review, only a few examples will be highlighted. One of the early studies was reported by Yang et al. Their group profiled methylation status of 9 genes, namely GSTP1, SOCS1, CDH1, APC, p15, p16, p14, p73 and RAR-β in 51 HCC samples using methylation-specific polymerase chain reaction (MSP) [54]. Among these genes, methylation of SOCS1, APC and p15 were shown to be more frequently observed in HCV-positive HCC patients compared to HBV/HCV-negative HCC. Another group from Korea, Lee et al. examined the methylation level of CpG loci in 14 genes in sixty HCC paired samples and found that methylation of GSTP1 and CDH1 were associated with poorer overall survival [55]. Similarly, Yu et al. used MSP to identify methylation level of 24 genes in 28 HCC samples from a Chinese population. They successfully showed that

methylation of AR, DBCCR1, IRF7, OCT6, p73, and p16 were associated with late stage HCC [56]. These three studies laid a strong basis for subsequent methylation analysis. Many studies have since then attempted to associate clinical parameters with DNA methylation, particularly on genes that were validated in these three studies. Subsequent paragraphs will outline 4 of these genes, namely, p16, SOCS1, GSTP1 and CDH1 (Table 3).

Commonly studied methylation marker genes
p16 (CDKN2A) is one of the most reported genes that was shown to be hypermethylated and associated with clinical parameters in HCC. It is a tumor suppressor gene that plays a role in cell cycle regulation [57]. It was methylated in many other cancers as well [58]. Beside earlier study by Yu et al. [56], Shim et al. [59] and Su et al. [60] also reported that methylation level of p16 was associated with advanced stage of HCC. They showed that methylation of p16 gene increased from cirrhotic tissue to HCC. Studies have also shown that hepatitis virus positive HCC samples have higher p16 methylation compared to HCC with no viral infection [61-64]. Zhu et al. even further showed that HBx gene, a protein coded by HBV, was associated with methylation of p16 in HBV positive HCC samples [65]. Clearly, environment factors such as viral infection could possibly disturb the epigenetic profile of the liver and contribute towards carcinogenesis. In addition, vascular invasion [61] and tumor differentiation [66] were also shown to be associated with p16 methylation. As vascular invasiveness and tumor differentiation were both strong predictors of survival in HCC [67-69], it is not surprising that hypermethylation of p16 in HCC patients was shown to have worse disease free survival as well [70].

Another frequently studied prognostic marker is SOCS1 methylation. SOCS1 gene was shown to be negative regulator of JAK/STAT pathway and its suppression by hypermethylation promotes cell growth [71]. SOCS1 methylation was correlated with progression of HCC [72], age [73,74] and tumor size [73,75]. Moreover, as mentioned in earlier paragraph, SOCS1 methylation was shown by Yang et al. to be associated with HCV infected HCC [54]. Concurring their study, Nishida et al. [76] and Ko et al. [73] also revealed that SOCS1 methylation was more prevalent in HCV infected HCC compared to non-infected HCC. Interestingly, Chu et al. [75] and Okochi et al. [77] did not find this association to be significant in their studies; instead, they found liver cirrhosis in HCC to be closely linked to hypermethylation of SOCS1. As HCV infection may lead to liver cirrhosis [78], more studies are required to ascertain this pathological link between HCV infection, SOCS1 methylation and HCC progression.

GSTP1 belongs to Glutathione S-transferases family, where it plays a role in protecting cells against damage induced by carcinogens, and modulating signal transduction

Table 2 Genome wide methylation profiling in HCC

Promoter microarray

Discovery method	HCC patients (n)	Validation method	Validated genes	Publication	Year
MCAM-chip	10	Pyrosequencing	RASSF1A, p16, TBX4, MMP14, GNA14, SLC16A5, CCNA1	Gao et al. [36]	2008
	16	Pyrosequencing	KLHL35, PAX5, PENK, SPDYA, LINE-1	Shitani et al. [40]	2012
DMH-chip	21	MSP	KLK10, OXGR1	Lu et al. [37]	2008
MeDIP-chip	6	MassArray	CYP24A1, DLX1, ZNF141, RASGRF2, ZNF382, TUBB6, NPR1, RRAD, RUNX3, LOX, JAKMIP1, SFRP4, DUSP4, PARQ8, CYP7B1	Deng et al. [38]	2010
	11	Pyrosequencing	AKR1B10, CENPH, MMP9, MMP12, PAGE4, S100A5, MMP2, NUPR1	Stefanska et al. [39]	2011

Beadarray

Discovery method	HCC patients (n)	Validation method	Validated genes	Publication	Year
GoldenGate	20	Methylight assay	APC	Archer et al. [49]	2010
	38	Pyrosequencing	RASSF1, GSTP1, APC, GABRA5, LINE-1	Hernandez-Vargas et al. [43]	2010
	45	Bisulfite sequencing	ERG, HOXA9	Hou et al. [47]	2013
Infinium 27K	3	COBRA and bisulfite sequencing	WNK2, EMILIN2, TLX3, TM6SF1, TRIM58, HIST1H4F, GRASP	Tao et al. [44]	2011
	62	NIL	NIL	Yang et al. [118]	2011
	13	NIL	NIL	Ammerpohl et al. [48]	2012
	62	Pyrosequencing	CDKL2, STEAP4, HIST1H3G, CDKN2A, ZNF154	Shen et al. [42]	2012
	63	Pyrosequencing	PER3	Neumann et al. [50]	2012
	71	Pyrosequencing	NEFH, SMPD3	Revill et al. [53]	2013
Infinium 450K	66	NIL	NIL	Shen et al. [46]	2013
	27	Pyrosequencing	GSTP1, RASSF1, BMP4, DLGAP1, GPR35	Song et al. [45]	2013
	6	Bisulfite sequencing	DBX2, THY1	Zhang et.al. [41]	2013

COBRA, Combined bisulfite restriction analysis; *MSP*, Methylation specific PCR.

Table 3 Commonly studied methylation markers in HCC

Gene	HCC patients (n)	Clinicopathological correlation	Validation method	Publication	Year
p16	28	Tumor stage	MSP	Yu et al. [56]	2003
	18	Tumor stage	MSP	Shim et al. [59]	2003
	20	Tumor differentiation	MSP	Qin et al. [66]	2004
	50	Age, gender, virus infection (HBV/HCV)	MSP	Li et al. [62]	2004
	44	HBV infection	MSP	Jicai et al. [63]	2006
	60	Age, tumor stage, vascular invasion, virus infection (HBV/HCV)	MSP	Katoh et al. [61]	2006
	58	Tumor stage	MSP	Su et al. [60]	2007
	23	HBV infection (HBx)	MSP	Zhu et al. [65]	2007
	265	Disease free survival	MSP	Ko et al. [70]	2008
	118	Gender	Methylscreen	Wang et al. [119]	2012
SOCS1	50	Liver cirrhosis	MSP	Okochi et al. [77]	2003
	51	HCV infection	MSP	Yang et al. [54]	2003
	284	Age, tumor size, virus infection (HBV/HCV)	MSP	Ko et al. [73]	2008
	77	HCV infection	COBRA	Nishida et al. [76]	2008
	46	Liver cirrhosis, tumor size	MSP	Chu et al. [75]	2010
	46	Tumor stage	MethyLight	Um et al. [72]	2011
	29	Chemotherapy treatment	MSP	Saelee et al. [120]	2012
	116	Age and gender	Methylscreen	Zhang et al. [74]	2013
GSTP1	60	Overall survival	MSP	Lee et al. [55]	2003
	83	Alcohol consumption, gender	MSP	Zhang et al. [82]	2005
	60	Gender, viral infection (HBV/HCV)	MSP	Katoh et al. [61]	2006
	58	HBV infection, tumor stage	MSP	Su et al. [60]	2007
	77	HCV infection	COBRA	Nishida et al. [76]	2008
	166	HBV infection	Pyrosequencing	Lambert et al. [81]	2011
CDH1	60	Overall survival	MSP	Lee et al. [55]	2003
	32	Vascular invasion, recurrence	MSP	Ghee [91]	2005

COBRA, Combined Bisulfite Restriction Analysis; *HBV*, Hepatitis B virus; *HCV*, Hepatitis C virus; *HBx*, Hepatitis B virus X protein; *MSP*, Methylation-specific PCR.

pathways that control cell proliferation and cell death [79]. Promoter methylation of GSTP1 was first reported in prostatic carcinoma back in 1994 [80]. Since then, many groups reported such observation in other cancers, including HCC. Analogous to earlier mentioned two genes, GSTP1 was also found to be highly methylated in HCC infected with either HBV or HCV compared to non-infected HCC [60,61,76,81]. Interestingly, methylation of GSTP1 was significantly associated with gender [61,82] and alcohol intake [82]. Also, study by Lee et al. managed to show that patients with high GSTP1 methylation level have worse overall survival outcome [55]. Although many studies examined the association of GSTP1 methylation with clinicopathological characteristics, only a few found associations suggesting that GSTP1 methylation alone may not be sufficient to serve as good single prognostic predictor for HCC.

CDH1 is another well-known tumor suppressor gene that was found methylated in many cancers [83-88]. It was frequently methylated in HCC as well. Despite many studies showed that methylation of CDH1 was higher in HCC than adjacent non-tumors, it often was not significantly associated with clinical parameters [54,61,76,89,90]. Only Lee et al. reported that methylation of CDH1 was linked with worse overall survival [55], and Ghee et al. found that it was associated with vascular invasion and recurrence [91]. These reports suggest that probably CDH1 alone may not have the power to be an independent prognostic factor. Notably, the concurrent methylation of CDH1, GSTP1 and a few other genes were found to be significantly associated with levels of AFP, recurrence free survival (RFS) and tumor numbers (Table 4). This concordant methylation of a group of genes associated with specific tumor characteristics is known as CIMP or CpG island methylator phenotype [92]. Presently, many studies have attempted to elucidate CIMP in various cancers, including G-CIMP for gliomas [93], B-CIMP for breast cancer [94], and C-CIMP for colorectal cancer [95]. In HCC, concurrent methylation of various genes has been associated with various clinical phenotypes (Table 4) and

Table 4 CIMP studies in HCC

Genes used to define CIMP	HCC patients (n)	Clinicopathological correlation	Validation method	Publication	Year
ER, c-MYC, p14, p15, p16, p53, RB1, RASSF1A, WT1	50	AFP level	MSP	Zhang et al. [96]	2007
E2F1, p15, p16, p21, p27, p300, p53, RB, WT1	120	Metastasis	MSP	Zhang et al. [97]	2008
CDH1, p14, p15, p16, p21, RB1, RASSF1A, SYK, TIMP3, WT1	60	Metastasis, tumor stage	MSP	Cheng et al. [98]	2010
CDH1, DAPK, GSTP1, p16, SOCS1, SYK, XAF1	65	AFP, RFS, tumor numbers	MSP	Wu et al. [100]	2010
GSTP1, MGMT, OPCML, p14, p15, p16, p73, RARβ, SOCS1	115	OS, RFS	MSP	Li et al. [99]	2010
APC, CDH1, DKK, DLC1, RUNX3, SFRP1, WIF1	108	AFP level, DFS, Gender, HBV status, tumor stage	MSP	Liu et al. [101]	2011
APC, GSTP1, HIC1, p16, PRDM2, RASSF1A, RUNX3, SOCS1	177	DFS	Methylight	Nishida et al. [102]	2012

AFP, Alpha fetoprotein; *DFS*, Disease free survival; *HBV*, Hepatitis B virus; *MSP*, Methylation-specific PCR; *OS*, Overall survival; *RFS*, Recurrence free survival.

these could perhaps be known as CpG island methylator phenotype for hepatocellular carcinoma, or Hep-CIMP.

Hep-CIMP associated with clinicopathological parameters
Presently, almost all studies that attempt to characterize Hep-CIMP came from Chinese population and methylation level of these CIMP genes were determined by MSP method. L-X, Wei led a team that published three studies on Hep-CIMP from 2007 to 2010. They defined CIMP + as samples with five or more methylated marker genes. Interestingly, marker genes that they used to define CIMP status varied across three studies (Table 4). Nonetheless, they managed to associate CIMP status with elevated AFP level (AFP ≥ 30 μg/L) [96], tumor metastasis [97,98], telomerase activity [97], tumor–node–metastasis (TNM) staging and overall survival [98].

Li et al. on the other hand, examined the methylation status of nine marker genes, namely, p14, p15, p16, p73, GSTP1, MGMT, RARβ, SOCS-1, and OPCML in 115 tumors. They defined CIMP + as HCC samples with six or more such methylated genes. They showed that CIMP + patients with TNM stage I have significantly poorer recurrence-free survival (RFS) and overall survival (OS) compared to CIMP-, TNM stage I patients [99]. Wu et al. also reported similar observation, despite their definition of CIMP + differed slightly [100]. They considered a sample to be CIMP + as long as it has three or more methylated marker genes. Their study revealed that CIMP + patients have shorter RFS compared to CIMP- patients. On top of that, they also showed that tumor number and pre-operative AFP levels were significantly higher in CIMP + samples [100].

More recently, Liu et al. reported the CIMP status of 108 HCC tissues and plasma respectively, based on methylation level of seven marker genes (Table 4). They

found good concordance of methylation status between plasma and tissue samples. CIMP status in tumor tissues and plasma were both significantly associated with clinicopathological parameters such as AFP level, TNM staging, gender and HBV infection [101]. Lastly, Nishida et al. profiled methylation status of eight genes, namely, HIC1, SOCS1, GSTP1, p16, APC, RASSF1, PRDM2 and RUNX3, and found that these markers, collectively, were associated with shorter time-to-occurrence of HCC tumor [102]. Even though Nishida et al. did not report CIMP status in their study, their analysis was similar to the rest of the Hep-CIMP studies. Also worthy to note is that these markers were carefully selected to represent very early stage of HCC. This again emphasizes the potential clinical use of early CIMP + signature for diagnosis or prognosis purposes.

Currently, there is still no consensus on how we define CIMP for HCC. As mentioned previously, all studies determined CIMP status based on their own set of genes, even though we saw a few recurrent genes such as GSTP1 and p16. This is partly due to the technology limitation in the past as most studies used MSP for methylation profiling. With genome-wide profiling methods become more available, it is a matter of time that we can soon ascertain the methylation markers that make up Hep-CIMP.

DNA methylation as potential blood biomarker
HCC is associated with high mortality rate mainly due to late diagnosis [1]. Therefore, there is an urgent need to identify promising tool that could diagnose the disease early or be served as surveillance for patients at risk. DNA methylation profile derived from blood samples could potentially be such biomarker. The attempt to identify methylation marker in blood dated back as early as 2003, where Wong et al. used quantitative MSP to measure the

Table 5 Methylation studies on DNA extracted from HCC blood samples

With clinicopathological correlation

Marker genes	HCC patients (n)	Clinicopathological correlation	Validation method	Samples used for DNA extraction	Publication	Year
DAPK, p16	64	AFP level	MSP	Serum	Lin et al. [104]	2005
RASSF1A	40	Tumor size	MSP	Plasma	Yeo et al. [106]	2005
LINE-1	85	OS, tumor size	COBRA	Serum	Tangkijvanich et al. [109]	2007
RASSF1A	85	DFS	Methylscreen	Serum	Chan et al. [107]	2008
CCND2	70	DFS	qMSP	Serum	Tsutsui et al. [105]	2010
APC, DKK, DLC1, CDH1, RUNX3, SFRP1, WIF1	108	CIMP + associated with gender, HBV infection, AFP level, tumor stage, DFS	MSP	Plasma	Liu et al. [101]	2011
APC, GSTP1, RASSF1A, SFRP1	72	OS (APC, RASSF1A)	Methylscreen	Plasma	Huang et al. [108]	2011
LINE-1	305	Increased risk of HCC	Pyrosequencing	White blood cells	Wu et al. [110]	2012
IGFBP7	136	Vascular invasion	MSP	Serum	Li et al. [115]	2013
XPO4	44	AFP level	MSP	PBMC	Zhang et al. [114]	2013
TFPI2	43	Tumor stage	MSP	Serum	Sun et al. [113]	2013
APC	23	Portal vein thrombosis	qMSP	Serum	Nishida et al. [112]	2013

Without clinicopathological correlation

Marker genes	HCC patients (n)	Clinicopathological correlation	Validation method	Samples used for DNA extraction	Publication	Year
p16	29	NIL	qMSP	Serum and buffy coat	Wong et al. [103]	2003
p16	46	NIL	MSP	Serum	Chu et al. [121]	2004
GSTP1	32	NIL	MSP	Serum	Wang et al. [122]	2006
CDH1, p16, RASSF1A, RUNX3	8	NIL	MSP	Serum	Tan et al. [123]	2007
p16, p15, RASSF1A	50	NIL	MSP	Serum	Zhang et al. [124]	2007
GSTP1, RASSF1A	26	NIL	MSP	Serum	Chang et al. [125]	2008
RASSF1A	35	NIL	MSP	Serum	Hu et al. [126]	2010
APC, CDH1, FHIT, p15, p16	28	NIL	MSP	Plasma	Iyer et al. [127]	2010
DBX2, THY1	31	NIL	Bisulfite seq	PBMC	Zhang et al. [41]	2013

AFP, Alpha fetoprotein; *COBRA*, Combined Bisulfite Restriction Analysis; *DFS*, Disease free survival; *HBV*, Hepatitis B virus; *MSP*, Methylation-specific PCR; *qMSP*, Quantitative MSP; *OS*, Overall survival; *PBMC*, Peripheral blood mononuclear cells; *Seq*, Sequencing.

methylation status of p16 in 29 HCC patients [103]. However, they did not perform any clinical association with their data. In fact, many studies successfully measure the aberrant methylation level of a marker gene in blood but did not associate it with clinicopathological parameters. These studies can be found in Table 5.

In this section, we will only highlight reports with significant clinical association. As shown in Table 5, Lin et al. showed that among 64 patients, about 77% of them have p16 methylation and 41% of them have DAPK methylation. Both markers were associated with AFP levels but no other parameters [104]. Tsutsui et al. found that the serum of 39 out of 70 patients were positive for methylated CCND2 gene. Patients of this group had shorter disease free survival [105].

Yeo et al. used MSP and found that 17 out of 40 patients' plasma (42.5%) had RASSF1A hypermethylation and their methylation status was associated with tumor size [106]. Chan et al. used another method, methylation-sensitive restriction enzyme-mediated real-time PCR system, to detect RASSF1A methylation status in 85 HCC sera. They found that 93% of them have hypermethylation and their methylated status was associated with shorter disease free survival and time-to-occurrence for HCC [107]. Using similar detection method, Huang et al. also showed that RASSF1A gene in 72 patients' blood was hypermethylated compared to normal controls and that its methylation level was associated with poorer overall survival [108].

Tangkijvanich et al. used Combined Bisulfite Restriction Analysis (COBRA) method to measure the hypomethylation of LINE-1 in 85 patients' sera. They reported that hypomethylation of LINE-1 was associated with HBV infection, larger tumor size and more advance disease stage [109]. Their study was further validated by Wu et al., where they used pyrosequencing to determine the methylation level of LINE-1 in 305 patients' white blood cell DNA [110]. They used logistic regression model to show that hypomethylation of LINE-1 increased overall risk of developing HCC. Recently, Gao et al. also reported that LINE-1 was hypomethylated in 71 HCC tissues and was associated with poorer prognosis [111]. These are few studies that showed hypomethylation instead of hypermethylation as potential prognostic biomarker.

Following the availability of genome-wide methylation profile, we also saw a sudden surge of methylation studies based on patients' sera. Within year 2013, four studies reported prognostic value of four different hypermethylated genes. Briefly, Nishida et al. performed quantitative MSP and showed that APC was more methylated in 23 HCC sera compared to healthy volunteers. They also showed that patients with higher APC methylation were associated with portal vein thrombosis [112]. Sun et al. detected TFPI2 to be more methylated in 43 HCC sera and its level

was associated with TNM stage [113]. Zhang et al. on the other hand found XPO4 to be frequently methylated in 44 patients' peripheral blood mononuclear cells. Their data indicated that higher XPO4 methylation was associated with higher AFP level [114]. Lastly, Li et al. discovered that in HBV-associated HCC, IGFBP7 was more methylated compared to chronic hepatitis B patients and normal controls. Also, its methylation status was associated with vascular invasion in HCC [115].

Clearly, methylation of marker gene in HCC blood DNA has potential prognostic value as shown by its association with clinicopathological data. However, most current studies drawn conclusion from a retrospective cohort. In order to translate these markers into actual clinical use, proper prospective studies and validation method are required.

Conclusions

Many genome wide methylation studies have confirmed that HCC has distinct methylation profile (Table 2), and some even showed that it is associated with different etiological factors such as HBV infection and alcohol consumption. Undeniably, the availability of these genome wide data has allowed the discovery of many novel genes with aberrant methylation, especially in recent years. As shown in Additional file 1: Table S1, apart from the commonly studied genes mentioned in this review, there is plethora of genes that were differentially methylated and associated with clinicopathological data. Future studies need to focus on collating current available data, shortlisting potential methylation markers by conducting proper validation method [116,117] and defining well-characterized CIMP status of HCC. It is hoped that emerging methylation markers can be used as diagnostic or prognostic marker for HCC in near future.

Additional file

Additional file 1: Table S1. List of methylation studies on HCC from year 2003–2013.

Author details

[1]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore. [2]Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, National Cancer Centre Singapore, Level 6, Lab 5, 11 Hospital Drive, Singapore 169610, Singapore. [3]NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore 117456, Singapore. [4]Duke-NUS Graduate Medical School, Singapore 169857, Singapore.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: **Global cancer statistics.** *CA Cancer J Clin* 2011, **61**(2):69–90.
2. Villanueva A, Minguez B, Forner A, Reig M, Llovet JM: **Hepatocellular carcinoma: novel molecular approaches for diagnosis, prognosis, and therapy.** *Annu Rev Med* 2010, **61**:317–328.
3. Hoofnagle JH: **Hepatocellular carcinoma: summary and recommendations.** *Gastroenterology* 2004, **127**(5 Suppl 1):S319–S323.
4. Yang JD, Roberts LR: **Hepatocellular carcinoma: a global view.** *Nat Rev Gastroenterol Hepatol* 2010, **7**(8):448–458.
5. Poon D, Anderson BO, Chen LT, Tanaka K, Lau WY, van Cutsem E, Singh H, Chow WC, Ooi LL, Chow P, Khin MW, Koo WH: **Management of hepatocellular carcinoma in Asia: consensus statement from the Asian Oncology Summit 2009.** *Lancet Oncol* 2009, **10**(11):1111–1118.
6. Colli A, Fraquelli M, Casazza G, Massironi S, Colucci A, Conte D, Duca P: **Accuracy of ultrasonography, spiral CT, magnetic resonance, and alpha-fetoprotein in diagnosing hepatocellular carcinoma: a systematic review: CME.** *Am J Gastroenterol* 2006, **101**(3):513–523.
7. Terentiev AA, Moldogazieva NT: **Alpha-fetoprotein: a renaissance.** *Tumour Biol* 2013, **34**(4):2075–2091.
8. Song P, Tobe RG, Inagaki Y, Kokudo N, Hasegawa K, Sugawara Y, Tang W: **The management of hepatocellular carcinoma around the world: a comparison of guidelines from 2001 to 2011.** *Liver Int* 2012, **32**(7):1053–1063.
9. Llovet JM, Bru C, Bruix J: **Prognosis of hepatocellular carcinoma: the BCLC staging classification.** *Semin Liver Dis* 1999, **19**(3):329–338.
10. Borel F, Konstantinova P, Jansen PLM: **Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma.** *J Hepatol* 2012, **56**(6):1371–1383.
11. Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, Tang ZY, Xin WW: **Identification of metastasis-related microRNAs in hepatocellular carcinoma.** *Hepatology* 2008, **47**(3):897–907.
12. Hoshida Y, Nijman SMB, Kobayashi M, Chan JA, Brunet JP, Chiang DY, Villanueva A, Newell P, Ikeda K, Hashimoto M, Watanabe G, Gabriel S, Friedman SL, Kumada H, Llovet JM, Golub TR: **Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma.** *Cancer Res* 2009, **69**(18):7385–7392.
13. Lee JS, Thorgeirsson SS: **Genome-scale profiling of gene expression in hepatocellular carcinoma: classification, survival prediction, and identification of therapeutic targets.** *Gastroenterology* 2004, **127**(SUPPL):S51–S55.
14. Lemmer ER, Friedman SL, Llovet JM: **Molecular diagnosis of chronic liver disease and hepatocellular carcinoma: the potential of gene expression profiling.** *Semin Liver Dis* 2006, **26**(4):373–384.
15. Villanueva A, Hoshida Y, Battiston C, Tovar V, Sia D, Alsinet C, Cornella H, Liberzon A, Kobayashi M, Kumada H, Thung SN, Bruix J, Newell P, April C, Fan J, Roayaie S, Mazzaferro V, Schwartz ME, Llovet JM: **Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma.** *Gastroenterology* 2011, **140**(5):1501–1512. e1502.
16. Minouchi K, Kaneko S, Kobayashi K: **Mutation of p53 gene in regenerative nodules in cirrhotic liver.** *J Hepatol* 2002, **37**(2):231–239.
17. Bressac B, Kew M, Wands J, Ozturk M: **Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa.** *Nature* 1991, **350**(6317):429–431.
18. Ishizaki Y, Ikeda S, Fujimori M, Shimizu Y, Kurihara T, Itamoto T, Kikuchi A, Okajima M, Asahara T: **Immunohistochemical analysis and mutational analyses of beta-catenin, Axin family and APC genes in hepatocellular carcinomas.** *Int J Oncol* 2004, **24**(5):1077–1083.
19. Edamoto Y, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle HM, Matsuda M, Fujii H, Scoazec JY, Ohgaki H: **Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis.** *Int J Cancer* 2003, **106**(3):334–341.
20. Satoh S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, Murata M, Shimano T, Yamaoka Y, Nakamura Y: **AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1.** *Nat Genet* 2000, **24**(3):245–250.
21. Taniguchi K, Roberts LR, Aderca IN, Dong X, Qian C, Murphy LM, Nagorney DM, Burgart LJ, Roche PC, Smith DI, Ross JA, Liu W: **Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas.** *Oncogene* 2002, **21**(31):4863–4871.
22. Han ZG: **Functional genomic studies: insights into the pathogenesis of liver cancer.** *Annu Rev Genom Hum G* 2012, **13**:171–205.
23. Pradhan S, Bacolla A, Wells RD, Roberts RJ: **Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation.** *J Biol Chem* 1999, **274**(46):33002–33010.
24. Feinberg AP, Vogelstein B: **Hypomethylation distinguishes genes of some human cancers from their normal counterparts.** *Nature* 1983, **301**(5895):89–92.
25. Tischoff I, Tannapfel A: **DNA methylation in hepatocellular carcinoma.** *World J Gastroentero* 2008, **14**(11):1741–1748.
26. Park IY, Sohn BH, Yu E, Suh DJ, Chung Y, Lee J, Surzycki SJ, Lee YI: **Aberrant epigenetic modifications in hepatocarcinogenesis induced by hepatitis B virus X protein.** *Gastroenterology* 2007, **132**(4):1476–1494.
27. Lim JS, Park SH, Jang KL: **Hepatitis C virus core protein overcomes stress-induced premature senescence by down-regulating p16 expression via DNA methylation.** *Cancer Lett* 2012, **321**(2):154–161.
28. Arora P, Kim EO, Jung JK, Jang KL: **Hepatitis C virus core protein downregulates E-cadherin expression via activation of DNA methyltransferase 1 and 3b.** *Cancer Lett* 2008, **261**(2):244–252.
29. Nagai M, Nakamura A, Makino R, Mitamura K: **Expression of DNA (5-cytosin)-methyltransferases (DNMTs) in hepatocellular carcinomas.** *Hepatol Res* 2003, **26**(3):186–191.
30. Park HJ, Yu E, Shim YH: **DNA methyltransferase expression and DNA hypermethylation in human hepatocellular carcinoma.** *Cancer Lett* 2006, **233**(2):271–278.
31. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R, Gunderson KL: **Genome-wide DNA methylation profiling using Infinium(R) assay.** *Epigenomics* 2009, **1**(1):177–200.
32. Touleimat N, Tost J: **Complete pipeline for Infinium((R)) Human Methylation 450 K BeadChip data processing using subset quantile normalization for accurate DNA methylation estimation.** *Epigenomics* 2012, **4**(3):325–341.
33. Estécio MRH, Yan PS, Ibrahim AEK, Tellez CS, Shen L, Huang THM, Issa JPJ: **High-throughput methylation profiling by MCA coupled to CpG island microarray.** *Genome Res* 2007, **17**(10):1529–1536.
34. Huang TH, Perry MR, Laux DE: **Methylation profiling of CpG islands in human breast cancer cells.** *Hum Mol Genet* 1999, **8**(3):459–470.
35. Mohn F, Weber M, Schubeler D, Roloff TC: **Methylated DNA immunoprecipitation (MeDIP).** *Methods Mol Biol* 2009, **507**:55–64.
36. Gao W, Kondo Y, Shen L, Shimizu Y, Sano T, Yamao K, Natsume A, Goto Y, Ito M, Murakami H, Osada H, Zhang J, Issa JPJ, Sekido Y: **Variable DNA methylation patterns associated with progression of disease in hepatocellular carcinomas.** *Carcinogenesis* 2008, **29**(10):1901–1910.
37. Lu CY, Hsieh SY, Lu YJ, Wu CS, Chen LC, Lo SJ, Wu CT, Chou MY, Huang THM, Chang YS: **Aberrant DNA methylation profile and frequent methylation of KLK10 and OXGR1 genes in hepatocellular carcinoma.** *Genes Chromosomes Cancer* 2009, **48**(12):1057–1068.
38. Deng YB, Nagae G, Midorikawa Y, Yagi K, Tsutsumi S, Yamamoto S, Hasegawa K, Kokudo N, Aburatani H, Kaneda A: **Identification of genes

preferentially methylated in hepatitis C virus-related hepatocellular carcinoma. *Cancer Sci* 2010, **101**(6):1501–1510.

39. Stefanska B, Huang J, Bhattacharyya B, Suderman M, Hallett M, Han ZG, Szyf M: Definition of the landscape of promoter DNA hypomethylation in liver cancer. *Cancer Res* 2011, **71**(17):5891–5903.
40. Shitani M, Sasaki S, Akutsu N, Takagi H, Suzuki H, Nojima M, Yamamoto H, Tokino T, Hirata K, Imai K, Toyota M, Shinomura Y: Genome-wide analysis of DNA methylation identifies novel cancer-related genes in hepatocellular carcinoma. *Tumor Biol* 2012, **33**(5):1307–1317.
41. Zhang P, Wen X, Gu F, Deng X, Li J, Dong J, Jiao J, Tian Y: Methylation profiling of serum DNA from hepatocellular carcinoma patients using an Infinium Human Methylation 450 BeadChip. *Hepatol Int* 2013, **7**(3):893–900.
42. Shen J, Wang S, Zhang YJ, Kappil M, Wu HC, Kibriya MG, Wang Q, Jasmine F, Ahsan H, Lee PH, Yu MW, Chen CJ, Santella RM: Genome-wide DNA methylation profiles in hepatocellular carcinoma. *Hepatology* 2012, **55**(6):1799–1808.
43. Hernandez-Vargas H, Lambert MP, le Calvez-Kelm F, Gouysse G, McKay-Chopin S, Tavtigian SV, Scoazec JY, Herceg Z: Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. *PLoS One* 2010, **5**(3):e9749.
44. Tao R, Li J, Xin J, Wu J, Guo J, Zhang L, Jiang L, Zhang W, Yang Z, Li L: Methylation profile of single hepatocytes derived from hepatitis B virus-related hepatocellular carcinoma. *PLoS One* 2011, **6**(5):e19862.
45. Song MA, Tiirikainen M, Kwee S, Okimoto G, Yu H, Wong LL: Elucidating the landscape of aberrant DNA methylation in hepatocellular carcinoma. *PLoS One* 2013, **8**(2):e55761.
46. Shen J, Wang S, Zhang YJ, Wu HC, Kibriya MG, Jasmine F, Ahsan H, Wu DPH, Siegel AB, Remotti H, Santella RM: Exploring genome-wide DNA methylation profiles altered in hepatocellular carcinoma using Infinium Human Methylation 450 BeadChips. *Epigenetics* 2013, **8**(1):34–43.
47. Hou X, Peng JX, Hao XY, Cai JP, Liang LJ, Zhai JM, Zhang KS, Lai JM, Yin XY: DNA methylation profiling identifies EYA4 gene as a prognostic molecular marker in hepatocellular carcinoma. *Ann Surg Oncol* 2013. in press.
48. Ammerpohl O, Pratschke J, Schafmayer C, Haake A, Faber W, von Kampen O, Brosch M, Sipos B, von Schönfels W, Balschun K, Röcken C, Arlt A, Schniewind B, Grauholm J, Kalthoff H, Neuhaus P, Stickel F, Schreiber S, Becker T, Siebert R, Hampe J: Distinct DNA methylation patterns in cirrhotic liver and hepatocellular carcinoma. *Int J Cancer* 2012, **130**(6):1319–1328.
49. Archer KJ, Mas VR, Maluf DG, Fisher RA: High-throughput assessment of CpG site methylation for distinguishing between HCV-cirrhosis and HCV-associated hepatocellular carcinoma. *Mol Genet Genomics* 2010, **283**(4):341–349.
50. Neumann O, Kesselmeier M, Geffers R, Pellegrino R, Radlwimmer B, Hoffmann K, Ehemann V, Schemmer P, Schirmacher P, Bermejo JL, Longerich T: Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors. *Hepatology* 2012, **56**(5):1817–1827.
51. Matsumura S, Imoto I, Kozaki KI, Matsui T, Muramatsu T, Furuta M, Tanaka S, Sakamoto M, Arii S, Inazawa J: Integrative array-based approach identifies MZB1 as a frequently methylated putative tumor suppressor in hepatocellular carcinoma. *Clin Cancer Res* 2012, **18**(13):3541–3551.
52. Okamura Y, Nomoto S, Hayashi M, Hishida M, Nishikawa Y, Yamada S, Fujii T, Sugimoto H, Takeda S, Kodera Y, Nakao A: Identification of the bleomycin hydrolase gene as a methylated tumor suppressor gene in hepatocellular carcinoma using a novel triple-combination array method. *Cancer Lett* 2011, **312**(2):150–157.
53. Revill K, Wang T, Lachenmayer A, Kojima K, Harrington A, Li J, Hoshida Y, Llovet JM, Powers S: Genome-wide methylation analysis and epigenetic unmasking identify tumor suppressor genes in hepatocellular carcinoma. *Gastroenterology* 2013, **145**(6):1424–1435.
54. Yang B, Guo M, Herman JG, Clark DP: Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. *Am J Pathol* 2003, **163**(3):1101–1107.
55. Lee S, Lee HJ, Kim JH, Lee HS, Jang JJ, Kang GH: Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. *Am J Pathol* 2003, **163**(4):1371–1378.
56. Yu J, Zhang HY, Ma ZZ, Lu W, Wang YF, Zhu J: Methylation profiling of twenty four genes and the concordant methylation behaviours of

57. nineteen genes that may contribute to hepatocellular carcinogenesis. *Cell Res* 2003, **13**(5):319–333.
57. Liggett WH, Sidransky D: Role of the p16 tumor suppressor gene in cancer. *J Clin Oncol* 1998, **16**(3):1197–1206.
58. Esteller M, Corn PG, Baylin SB, Herman JG: A gene hypermethylation profile of human cancer. *Cancer Res* 2001, **61**(8):3225–3229.
59. Shim YH, Yoon GS, Choi HJ, Chung YH, Yu E: p16 Hypermethylation in the early stage of hepatitis B virus-associated hepatocarcinogenesis. *Cancer Lett* 2003, **190**(2):213–219.
60. Su PF, Lee TC, Lin PJ, Lee PH, Jeng YM, Chen CH, Liang JD, Chiou LL, Huang GT, Lee HS: Differential DNA methylation associated with hepatitis B virus infection in hepatocellular carcinoma. *Int J Cancer* 2007, **121**(6):1257–1264.
61. Katoh H, Shibata T, Kokubu A, Ojima H, Fukayama M, Kanai Y, Hirohashi S: Epigenetic instability and chromosomal instability in hepatocellular carcinoma. *Am J Pathol* 2006, **168**(4):1375–1384.
62. Li X, Hei AM, Sun L, Hasegawa K, Torzilli G, Minagawa M, Takayama T, Makuuchi M: p16INK4A hypermethylation is associated with hepatitis virus infection, age, and gender in hepatocellular carcinoma. *Clin Cancer Res* 2004, **10**(22):7484–7489.
63. Jicai Z, Zongtao Y, Jun L, Haiping L, Jianmin W, Lihua H: Persistent infection of hepatitis B virus is involved in high rate of p16 methylation in hepatocellular carcinoma. *Mol Carcinog* 2006, **45**(7):530–536.
64. Feng Q, Stern JE, Hawes SE, Lu H, Jiang M, Kiviat NB: DNA methylation changes in normal liver tissues and hepatocellular carcinoma with different viral infection. *Exp Mol Pathol* 2010, **88**(2):287–292.
65. Zhu R, Li BZ, Li H, Ling YQ, Hu XQ, Zhai WR, Zhu HG: Association of p16INK4A hypermethylation with hepatitis B virus X protein expression in the early stage of HBV-associated hepatocarcinogenesis. *Pathol Int* 2007, **57**(6):328–336.
66. Qin Y, Liu JY, Li B, Sun ZL, Sun ZF: Association of low p16INK4a and p15INK4b mRNAs expression with their CpG islands methylation with human hepatocellular carcinogenesis. *World J Gastroenterol* 2004, **10**(9):1276–1280.
67. Kikuchi LO, Paranagua-Vezozzo DC, Chagas AL, Mello ES, Alves VA, Farias AQ, Pietrobon R, Carrilho FJ: Nodules less than 20 mm and vascular invasion are predictors of survival in small hepatocellular carcinoma. *J Clin Gastroenterol* 2009, **43**(2):191–195.
68. Tamura S, Kato T, Berho M, Misiakos EP, O'Brien C, Reddy KR, Nery JR, Burke GW, Schiff ER, Miller J, Tzakis AG: Impact of histological grade of hepatocellular carcinoma on the outcome of liver transplantation. *Arch Surg* 2001, **136**(1):25–30.
69. Jonas S, Bechstein WO, Steinmuller T, Herrmann M, Radke C, Berg T, Settmacher U, Neuhaus P: Vascular invasion and histopathologic grading determine outcome after liver transplantation for hepatocellular carcinoma in cirrhosis. *Hepatology* 2001, **33**(5):1080–1086.
70. Ko E, Kim Y, Kim SJ, Joh JW, Song S, Park CK, Park J, Kim DH: Promoter hypermethylation of the p16 gene is associated with poor prognosis in recurrent early-stage hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev* 2008, **17**(9):2260–2267.
71. Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, Harris CC, Herman JG: SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat Genet* 2001, **28**(1):29–35.
72. Um TH, Kim H, Oh BK, Kim MS, Kim KS, Jung G, Park YN: Aberrant CpG island hypermethylation in dysplastic nodules and early HCC of hepatitis B virus-related human multistep hepatocarcinogenesis. *J Hepatol* 2011, **54**(5):939–947.
73. Ko E, Kim SJ, Joh JW, Park CK, Park J, Kim DH: CpG island hypermethylation of SOCS-1 gene is inversely associated with HBV infection in hepatocellular carcinoma. *Cancer Lett* 2008, **271**(2):240–250.
74. Zhang X, Wang J, Cheng J, Ding S, Li M, Sun S, Zhang L, Liu S, Chen X, Zhuang H, Lu F: An integrated analysis of SOCS1 down-regulation in HBV infection-related hepatocellular carcinoma. *J Viral Hepat* 2013. in press.
75. Chu PY, Yeh CM, Hsu NC, Chang YS, Chang JG, Yeh KT: Epigenetic alteration of the SOCS1 gene in hepatocellular carcinoma. *Swiss Med Wkly* 2010, **140**.
76. Nishida N, Nagasaka T, Nishimura T, Ikai I, Boland CR, Goel A: Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. *Hepatology* 2008, **47**(3):908–918.

77. Okochi O, Hibi K, Sakai M, Inoue S, Takeda S, Kaneko T, Nakao A: Methylation-mediated silencing of SOCS-1 gene in hepatocellular carcinoma derived from cirrhosis. *Clin Cancer Res* 2003, **9**(14):5295–5298.

78. Freeman AJ, Dore GJ, Law MG, Thorpe M, von Overbeck J, Lloyd AR, Marinos G, Kaldor JM: Estimating progression to cirrhosis in chronic hepatitis C virus infection. *Hepatology* 2001, **34**(4 Pt 1):809–816.

79. Laborde E: Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. *Cell Death Differ* 2010, **17**(9):1373–1380.

80. Lee WH, Morton RA, Epstein JI, Brooks JD, Campbell PA, Bova GS, Hsieh WS, Isaacs WB, Nelson WG: Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc Natl Acad Sci USA* 1994, **91**(24):11733–11737.

81. Lambert MP, Paliwal A, Vaissière T, Chemin I, Zoulim F, Tommasino M, Hainaut P, Sylla B, Scoazec JY, Tost J, Herceg Z: Aberrant DNA methylation distinguishes hepatocellular carcinoma associated with HBV and HCV infection and alcohol intake. *J Hepatol* 2011, **54**(4):705–715.

82. Zhang YJ, Chen Y, Ahsan H, Lunn RM, Chen SY, Lee PH, Chen CJ, Santella RM: Silencing of glutathione S-transferase P1 by promoter hypermethylation and its relationship to environmental chemical carcinogens in hepatocellular carcinoma. *Cancer Lett* 2005, **221**(2):135–143.

83. Chang HW, Chow V, Lam KY, Wei WI, WingYuen AP: Loss of E-cadherin expression resulting from promoter hypermethylation in oral tongue carcinoma and its prognostic significance. *Cancer* 2002, **94**(2):386–392.

84. Kanazawa T, Watanabe T, Kazama S, Tada T, Koketsu S, Nagawa H: Poorly differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum show higher rates of loss of heterozygosity and loss of E-cadherin expression due to methylation of promoter region. *Int J Cancer* 2002, **102**(3):225–229.

85. Graziano F, Arduini F, Ruzzo A, Bearzi I, Humar B, More H, Silva R, Muretto P, Guilford P, Testa E, Mari D, Magnani M, Cascinu S: Prognostic analysis of E-cadherin gene promoter hypermethylation in patients with surgically resected, node-positive, diffuse gastric cancer. *Clin Cancer Res* 2004, **10**(8):2784–2789.

86. Takeno S, Noguchi T, Fumoto S, Kimura Y, Shibata T, Kawahara K: E-cadherin expression in patients with esophageal squamos cell carcinoma: Promoter hypermethylation, Snail overexpression, and clinicopathologic implications. *Am J Clin Pathol* 2004, **122**(1):78–84.

87. Shimamoto T, Ohyashiki JH, Ohyashiki K: Methylation of p15INK4b and E-cadherin genes is independently correlated with poor prognosis in acute myeloid leukemia. *Leuk Res* 2005, **29**(6):653–659.

88. Caldeira JRF, Prando EC, Quevedo FC, Moraes Neto FA, Rainho CA, Rogatto SR: CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer. *BMC Cancer* 2006, **6**:48.

89. Lim SO, Gu JM, Kim MS, Kim HS, Park YN, Park CK, Cho JW, Park YM, Jung G: Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. *Gastroenterology* 2008, **135**(6):2128–2140.

90. Oh BK, Kim H, Park HJ, Shim YH, Choi J, Park C, Park YN: DNA methyltransferase expression and DNA methylation in human hepatocellular carcinoma and their clinicopathological correlation. *Int J Mol Med* 2007, **20**(1):65–73.

91. Ghee YK, Byung CY, Kwang CK, Jae WC, Won SP, Cheol KP: Promoter methylation of E-cadherin in hepatocellular carcinomas and dysplastic nodules. *J Korean Med Sci* 2005, **20**(2):242–247.

92. Hughes LA, Melotte V, de Schrijver J, de Maat M, Smit VT, Bovee JV, French PJ, van den Brandt PA, Schouten LJ, de Meyer T, van Criekinge W, Ahuja N, Herman JG, Weijenberg MP, van Engeland M: The CpG island methylator phenotype: what's in a name? *Cancer Res* 2013, **73**(19):5858–5868.

93. Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, Pan F, Pelloski CE, Sulman EP, Bhat KP, Verhaak RG, Hoadley KA, Hayes DN, Perou CM, Schmidt HK, Ding L, Wilson RK, van den Berg D, Shen H, Bengtsson H, Neuvial P, Cope LM, Buckley J, Herman JG, Baylin SB, Laird PW, Aldape K: Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* 2010, **17**(5):510–522.

94. Fang F, Turcan S, Rimner A, Kaufman A, Giri D, Morris LG, Shen R, Seshan V, Mo Q, Heguy A, Baylin SB, Ahuja N, Viale A, Massague J, Norton L, Vahdat LT, Moynahan ME, Chan TA: Breast cancer methylomes establish an epigenomic foundation for metastasis. *Sci Transl Med* 2011, **3**(75):75ra25.

95. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP: CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999, **96**(15):8681–8686.

96. Zhang C, Li Z, Cheng Y, Jia F, Li R, Wu M, Li K, Wei L: CpG island methylator phenotype association with elevated serum α-fetoprotein level in hepatocellular carcinoma. *Clin Cancer Res* 2007, **13**(3):944–952.

97. Zhang C, Guo X, Jiang G, Zhang L, Yang Y, Shen F, Wu M, Wei L: CpG island methylator phenotype association with upregulated telomerase activity in hepatocellular carcinoma. *Int J Cancer* 2008, **123**(5):998–1004.

98. Cheng Y, Zhang C, Zhao J, Wang C, Xu Y, Han Z, Jiang G, Guo X, Li R, Bu X, Wu M, Wei L: Correlation of CpG island methylator phenotype with poor prognosis in hepatocellular carcinoma. *Exp Mol Pathol* 2010, **88**(1):112–117.

99. Li B, Liu W, Wang L, Li M, Wang J, Huang L, Huang P, Yuan Y: CpG island methylator phenotype associated with tumor recurrence in tumor-node-metastasis stage I hepatocellular carcinoma. *Ann Surg Oncol* 2010, **17**(7):1917–1926.

100. Wu LM, Zhang F, Zhou L, Yang Z, Xie HY, Zheng SS: Predictive value of CpG island methylator phenotype for tumor recurrence in hepatitis B virus-associated hepatocellular carcinoma following liver transplantation. *BMC Cancer* 2010, **10**:399.

101. Liu JB, Zhang YX, Zhou SH, Shi MX, Cai J, Liu Y, Chen KP, Qiang FL: CpG Island methylator phenotype in plasma is associated with hepatocellular carcinoma prognosis. *World J Gastroenterol* 2011, **17**(42):4718–4724.

102. Nishida N, Kudo M, Nagasaka T, Ikai I, Goel A: Characteristic patterns of altered DNA methylation predict emergence of human hepatocellular carcinoma. *Hepatology* 2012, **56**(3):994–1003.

103. Wong IHN, Zhang J, Lai PBS, Lau WY, Lo YMD: Quantitative analysis of tumor-derived methylated p16INK4a sequences in plasma, serum, and blood cells of hepatocellular carcinoma patients. *Clin Cancer Res* 2003, **9**(3):1047–1052.

104. Lin Q, Chen LB, Tang YM, Wang J: Promoter hypermethylation of p16 gene and DAPK gene in sera from hepatocellular carcinoma (HCC) patients. *Chin J Cancer Res* 2005, **17**(4):250–254.

105. Tsutsui M, Iizuka N, Moribe T, Miura T, Kimura N, Tamatsukuri S, Ishitsuka H, Fujita Y, Hamamoto Y, Tsunedomi R, Iida M, Tokuhisa Y, Sakamoto K, Tamesa T, Sakaida I, Oka M: Methylated cyclin D2 gene circulating in the blood as a prognosis predictor of hepatocellular carcinoma. *Clin Chim Acta* 2010, **411**(7–8):516–520.

106. Yeo W, Wong N, Wong WL, Lai PBS, Zhong S, Johnson PJ: High frequency of promoter hypermethylation of RASSF1A in tumor and plasma of patients with hepatocellular carcinoma. *Liver Int* 2005, **25**(2):266–272.

107. Chan KCA, Lai PBS, Mok TSK, Chan HLY, Ding C, Yeung SW, Lo YMD: Quantitative analysis of circulating methylated DNA as a biomarker for hepatocellular carcinoma. *Clin Chem* 2008, **54**(9):1528–1536.

108. Huang ZH, Hu Y, Hua D, Wu YY, Song MX, Cheng ZH: Quantitative analysis of multiple methylated genes in plasma for the diagnosis and prognosis of hepatocellular carcinoma. *Exp Mol Pathol* 2011, **91**(3):702–707.

109. Tangkijvanich P, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, Mutirangura A: Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. *Clin Chim Acta* 2007, **379**(1–2):127–133.

110. Wu HC, Wang Q, Yang HI, Tsai W, Chen CJ, Santella RM: Global dna methylation levels in white blood cells as a biomarker for hepatocellular carcinoma risk: a nested case–control study. *Carcinogenesis* 2012, **33**(7):1340–1345.

111. Gao XD, Qu JH, Chang XJ, Lu YY, Bai WL, Wang H, Xu ZX, An LJ, Wang CP, Zeng Z, Yang YP: Hypomethylation of long interspersed nuclear element-1 promoter is associated with poor outcomes for curative resected hepatocellular carcinoma. *Liver Int* 2013. in press.

112. Nishida N, Arizumi T, Takita M, Nagai T, Kitai S, Yada N, Hagiwara S, Inoue T, Minami Y, Ueshima K, Sakurai T, Ida H, Kudo M: Quantification of tumor DNA in serum and vascular invasion of human hepatocellular carcinoma. *Oncology* 2013, **84**(SUPPL.1):82–87.

113. Sun FK, Fan YC, Zhao J, Zhang F, Gao S, Zhao ZH, Sun Q, Wang K: Detection of TFPI2 methylation in the serum of hepatocellular carcinoma patients. *Dig Dis Sci* 2013, **58**(4):1010–1015.

114. Zhang F, Fan YC, Mu NN, Zhao J, Sun FK, Zhao ZH, Gao S, Wang K: Exportin 4 gene expression and DNA promoter methylation status in chronic hepatitis B virus infection. *J Viral Hepat* 2013. in press.

115. Li F, Fan YC, Gao S, Sun FK, Yang Y, Wang K: **Methylation of serum insulin-like growth factor-binding protein 7 promoter in hepatitis B virus-associated hepatocellular carcinoma.** *Genes Chromosomes Cancer* 2014, **53**(1):90–97.

116. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM: **REporting recommendations for tumor MARKer prognostic studies (REMARK).** *Nat Clin Prac Urol* 2005, **2**(8):416–422.

117. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Lijmer JG, Moher D, Rennie D, de Vet HC: **Toward complete and accurate reporting of studies of diagnostic accuracy. The STARD initiative.** *Am J Clin Pathol* 2003, **119**(1):18–22.

118. Yang JD, Seol SY, Leem SH, Kim YH, Sun Z, Lee JS, Thorgeirsson SS, Chu IS, Roberts LR, Kang KJ: **Genes associated with recurrence of hepatocellular carcinoma: Integrated analysis by gene expression and methylation profiling.** *J Korean Med Sci* 2011, **26**(11):1428–1438.

119. Wang Y, Cheng J, Xu C, Liu S, Jiang S, Xu Q, Chen X, Zhuang H, Lu F: **Quantitative methylation analysis reveals gender and age differences in p16INK4a hypermethylation in hepatitis B virus-related hepatocellular carcinoma.** *Liver Int* 2012, **32**(3):420–428.

120. Saelee P, Chuensumran U, Wongkham S, Chariyalertsak S, Tiwawech D, Petmitr S: **Hypermethylation of suppressor of cytokine signaling 1 in hepatocellular carcinoma patients.** *Asian Pac J Cancer Prev* 2012, **13**(7):3489–3493.

121. Chu HJ, Heo J, Seo SB, Kim GH, Kang DH, Song GA, Cho M, Yang US: **Detection of aberrant p16INK4A methylation in sera of patients with liver cirrhosis and hepatocellular carcinoma.** *J Korean Med Sci* 2004, **19**(1):83–86.

122. Wang J, Qin Y, Li B, Sun Z, Yang B: **Detection of aberrant promoter methylation of GSTP1 in the tumor and serum of Chinese human primary hepatocellular carcinoma patients.** *Clin Biochem* 2006, **39**(4):344–348.

123. Tan SH, Ida H, Lau QC, Goh BC, Chieng WS, Loh M, Ito Y: **Detection of promoter hypermethylation in serum samples of cancer patients by methylation-specific polymerase chain reaction for tumour suppressor genes including RUNX3.** *Oncol Rep* 2007, **18**(5):1225–1230.

124. Zhang YJ, Wu HC, Shen J, Ahsan H, Wei YT, Yang HI, Wang LY, Chen SY, Chen CJ, Santella RM: **Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA.** *Clin Cancer Res* 2007, **13**(8):2378–2384.

125. Chang H, Yi B, Li L, Zhang HY, Sun F, Dong SQ, Cao Y: **Methylation of tumor associated genes in tissue and plasma samples from liver disease patients.** *Exp Mol Pathol* 2008, **85**(2):96–100.

126. Hu L, Chen G, Yu H, Qiu X: **Clinicopathological significance of RASSF1A reduced expression and hypermethylation in hepatocellular carcinoma.** *Hepatol Int* 2010, **4**(1):423–432.

127. Iyer P, Zekri AR, Hung CW, Schiefelbein E, Ismail K, Hablas A, Seifeldin IA, Soliman AS: **Concordance of DNA methylation pattern in plasma and tumor DNA of Egyptian hepatocellular carcinoma patients.** *Exp Mol Pathol* 2010, **88**(1):107–111.