

## Global hypomethylation in hepatocellular carcinoma and its relationship to aflatoxin B<sub>1</sub> exposure

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### Abstract

**AIM:** To determine global DNA methylation in paired hepatocellular carcinoma (HCC) samples using several different assays and explore the correlations between hypomethylation and clinical parameters and biomarkers, including that of aflatoxin B<sub>1</sub> exposure.

**METHODS:** Using the radio labeled methyl acceptance assay as a measure of global hypomethylation, as well as two repetitive elements, including satellite 2 (Sat2) by MethyLight and long interspersed nucleotide elements (LINE1), by pyrosequencing.

**RESULTS:** By all three assays, mean methylation levels in tumor tissues were significantly lower than that in

adjacent tissues. Methyl acceptance assay log (mean  $\pm$  SD) disintegrations/min/ng DNA are  $70.0 \pm 54.8$  and  $32.4 \pm 15.6$ , respectively,  $P = 0.040$ ; percent methylation of Sat2  $42.2 \pm 55.1$  and  $117.9 \pm 88.8$ , respectively,  $P < 0.0001$  and percent methylation LINE1  $48.6 \pm 14.8$  and  $71.7 \pm 1.4$ , respectively,  $P < 0.0001$ . Aflatoxin B<sub>1</sub>-albumin (AFB<sub>1</sub>-Alb) adducts, a measure of exposure to this dietary carcinogen, were inversely correlated with LINE1 methylation ( $r = -0.36$ ,  $P = 0.034$ ).

**CONCLUSION:** Consistent hypomethylation in tumor compared to adjacent tissue was found by the three different methods. AFB<sub>1</sub> exposure is associated with DNA global hypomethylation, suggesting that chemical carcinogens may influence epigenetic changes in humans.

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**Key words:** Hepatocellular carcinoma; Epigenetics; Hypomethylation; [<sup>3</sup>H]-methyl acceptance assay; Satellite 2; Long interspersed nucleotide element-1; Aflatoxin B<sub>1</sub>

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and a leading cause of death worldwide, especially in Saharan Africa and

southern Asia, including Taiwan, Thailand, Hong Kong and southern China<sup>[1,2]</sup>; it is also increasing in Western, developed countries such as the United States<sup>[3]</sup>. HCC incidence is associated with various risk factors, including chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcohol consumption and several environmental factors, especially aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), a dietary mold contaminant, and polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental contaminants<sup>[4-6]</sup>.

As with other cancers, the development of HCC is a complex, multistep process, involving multiple genetic aberrations in the molecular control of hepatocyte proliferation, differentiation and death and the maintenance of genomic integrity. This process is influenced by the cumulative activation and inactivation of oncogenes, tumor suppressor genes and other genes<sup>[7,8]</sup>. Epigenetic alterations are also involved in cancer development and progression<sup>[9,10]</sup>. Human tumors often display changes in DNA methylation, including both gene-specific promoter hypermethylation and genome-wide hypomethylation<sup>[11,12]</sup>. Frequent promoter hypermethylation and subsequent loss of protein expression of tumor suppressor genes has been demonstrated in HCC<sup>[13]</sup>. Global hypomethylation, in both noncoding repetitive sequences and in genes, contributes to carcinogenesis by causing chromosome instability, reactivation of transposable elements, loss of imprinting and increased gene expression, and has been detected in different human cancer tissues, including HCC<sup>[14]</sup>.

Hypomethylation of the genome mainly affects the intergenic and intronic regions of DNA, particularly repeat sequences and transposable elements<sup>[15]</sup>. Repetitive elements, which consist of interspersed and tandem repeats, comprise about 45% of the human genome<sup>[16,17]</sup>. More than 90% of all 5-methylcytosine (5<sup>m</sup>C) lies within the transposons, including short interspersed nucleotide elements and long interspersed nucleotide elements (LINEs), which are comparatively rich in CpG dinucleotides<sup>[14]</sup>. Satellite 2 (Sat2) DNA sequences are located as tandem repeats in the pericentromeric and juxtacentromeric heterochromatin of several chromosomes<sup>[18]</sup>. Loss of DNA methylation in these sequences is believed to mainly account for global hypomethylation<sup>[19]</sup>. Analysis of methylation levels of Sat2 and LINE1 are frequently used as a measure of global methylation since levels measured using the MethylLight assay were significantly associated with methylation, as measured by high-performance liquid chromatography quantitation of 5<sup>m</sup>C<sup>[20]</sup>.

The methyl group acceptance assay also can be used to determine global DNA methylation levels. It is based on the ability of isolated DNA to “accept” radio labeled methyl groups from S-[<sup>3</sup>H-methyl] adenosylmethionine, using the bacterial CpG methyltransferase SssI. As this enzyme methylates all unmethylated CpG dinucleotides in the genome, radio labeled methyl group acceptance is inversely proportional to the level of preexisting methylation<sup>[21]</sup>.

In the current study, global DNA methylation status in paired HCC and their adjacent non-tumor tissues was

measured using the methyl acceptance assay, analysis of Sat2 by MethylLight and LINE1 by pyrosequencing. Data were correlated to both clinical data and other available biomarker data on exposure to AFB<sub>1</sub> and gene-specific promoter methylation.

## MATERIALS AND METHODS

### Patient population and data on clinical parameters

The study samples consisted of frozen dissected tumor and adjacent tissues from HCC patients, collected in the Department of Surgery, National Taiwan University Hospital. Informed consent was obtained from patients and the study was approved by the appropriate institutional review committees. Data on demographics and clinicopathological characteristics obtained from hospital charts, and HBV and HCV status, determined by immunoassay, were published previously<sup>[22]</sup>. Plasma collected at the time of surgery had been previously analyzed for the albumin adducts of AFB<sub>1</sub>. In addition, methylation of *p16<sup>Ink4A</sup>* and *HINT1* were previously determined in the tumor tissues by methylation specific PCR<sup>[22-23]</sup>.

### DNA extraction

DNA was isolated from frozen tissue samples, as previously described<sup>[24]</sup>. Briefly, tissue was placed in liquid nitrogen and pulverized with a blender. The tissue powder was lysed with a DNA lysing buffer (10 mmol/L Tris, 10 mmol/L NaCl, 0.1% sodium dodecyl sulfate at pH 7.9 and 200 µg/mL proteinase K). DNA was isolated by RNase treatment, phenol/chloroform extraction and ethanol precipitation. The laboratory investigator who performed the assays was blinded to epidemiological data.

### Sat2 MethylLight assay

After sodium bisulfite conversion (EZ DNA methylation kit, Zymo Research, Orange, CA), genomic DNA was amplified using the previously reported Sat2 M1 and Alu C4 (control for DNA input) primers and probes<sup>[20]</sup>. Bisulfite-converted, CpGenome universal methylated DNA (Chemical International, Temecula, CA) served as the methylated reference. A pooled sample of DNA from 5 controls was used as a quality control and analyzed with each batch of test samples. All samples were analyzed in duplicate on an ABI Prism 7900 Sequence Detection System (Perkin-Elmer, Foster City, CA). Intra- and inter-assay coefficients of variation (CVs) were 1.2 and 1.9, respectively. The data are expressed as a percentage of methylated reference (PMR) values.

$$\text{PMR} = 100\% * 2^{\exp - \{ \Delta \text{Ct (target gene in sample)} - \Delta \text{Ct (target gene in fully methylated reference sample)} - \Delta \text{Ct (control gene in sample)} - \Delta \text{Ct (control gene in reference sample)} \} }.$$

### LINE1 amplification and pyrosequencing

The assay for LINE1 was carried out essentially as described previously, using reported primer and sequencing

**Table 1** Methylation levels of hepatocellular carcinoma tumor and adjacent non-tumor liver tissue

	Tumor mean $\pm$ SD	Adjacent mean $\pm$ SD	P value
LINE1 (%)	48.6 (14.8)	71.7 (1.4)	< 0.0001
Sat2 (%)	42.2 (55.1)	117.9 (88.8)	< 0.0001
Methyl acceptance (DPM/ng)	70.0 (54.8)	32.4 (15.6)	0.040

LINE1: Long interspersed nucleotide element-1; Sat2: Satellite 2; DPM: Disintegrations/min.

probe sequences as well as PCR conditions<sup>[25]</sup>. We used non-CpG cytosine residues as internal controls to verify efficient sodium bisulfate DNA conversion and controls were as in the MethyLight assay. Pyrosequencing was conducted using a PyroMark Q24 instrument (Qiagen), with subsequent quantitation of methylation levels determined with the PyroMark Q24 1.010 software. Relative peak height differences were used to calculate the percentage of methylated cytosines at each given site. Percent methylation within a sample was subsequently determined by averaging across all three interrogated CpG sites in the analysis. The inter-assay CV was 1.1.

### <sup>3</sup>H-Methyl acceptance assay

The [<sup>3</sup>H]-methyl acceptance assay was carried out as described by Balaghi and Wagner<sup>[26]</sup> and Pilsner *et al.*<sup>[27]</sup>. The DNA was incubated with [<sup>3</sup>H]S-adenosylmethionine in the presence of the SssI prokaryotic methylase enzyme. Briefly, 200 ng of DNA was incubated with 3 U of SssI methylase (New England Biolabs); 3.8  $\mu$ mol/L (1.1  $\mu$ Ci) [<sup>3</sup>H]-labeled S-adenosylmethionine (Perkin-Elmer); and EDTA, DTT, and Tris-HCL (pH 8.2) in a 30  $\mu$ L mixture and incubated for 1 h at 37 °C. The reaction was terminated on ice and 15  $\mu$ L of the reaction mixture applied onto Whatman DE81 filter paper. The filter was washed on a vacuum filtration apparatus three times with 5 mL of 0.5 mol/L sodium phosphate buffer (pH 8.0), followed by 2 mL each of 70% and 100% ethanol. Dried filters were each placed in a vial with 5 mL of scintillation fluid (Scintisafe, Thermo Fisher, Waltham, MA) and analyzed by a Packard scintillation counter to determine counts/min then converted to disintegrations/min (DPM) based on counting efficiency. Each DNA sample was processed in duplicate and each processing run included samples for background (reaction mixture with all components except SssI enzyme) and controls as for the other assays. Intra- and inter-assay CVs were 2.0 and 3.9, respectively. DPM values were expressed per ng DNA as quantified by PicoGreen using double-strand DNA quantification reagent (Molecular Probes, Life Technologies, Grand Island, NY).

### Statistical analysis

Paired *t*-test was used to examine differences in methylation levels between tumor and adjacent tissues after

**Table 2** Correlations between methylation levels and aflatoxin B<sub>1</sub>-albumin adducts in hepatocellular carcinoma tumor tissues

	AFB <sub>1</sub> -Alb <i>r</i>	P value
LINE1 (%)	-0.36	0.034
Sat2 (%)	-0.30	0.082
Methyl acceptance DPM/ng)	0.18	0.286

AFB<sub>1</sub>: Aflatoxin B<sub>1</sub>; LINE1: Long interspersed nucleotide element-1; Sat2: Satellite 2; DPM: Disintegrations/min.

natural log-transformation to normalize the distribution. We present the values as arithmetic data for ease of interpretation. Spearman correlation coefficients were used to determine the correlation between methylation and AFB<sub>1</sub>-Alb adducts. Wilcoxon signed-rank test was used to compare methylation levels and clinical characteristics. All analyses were performed with SAS software 9.0 (SAS Institute, Cary, NC). All statistical tests were based on two-tailed probability.

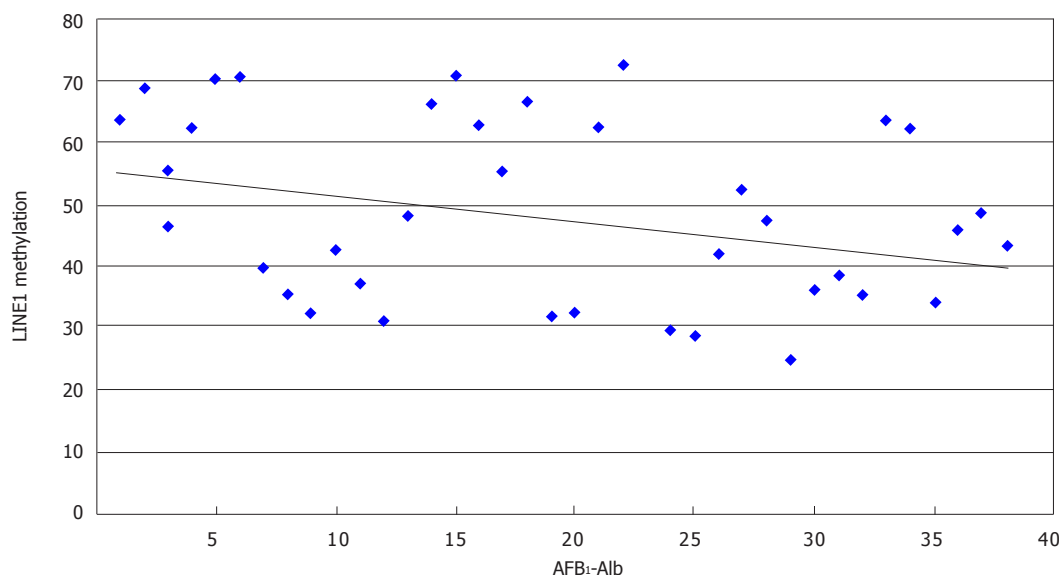
## RESULTS

Methylation levels of DNA from HCC and adjacent non-tumor tissues were determined by the methyl acceptance assay, a measure of global methylation. Two repetitive elements were also analyzed as an additional measure of methylation, including Sat2 by MethyLight and LINE1 by pyrosequencing. For all three assays, mean methylation level was significantly lower in tumor compared to adjacent non-tumor tissues. For the methyl acceptance assay, mean levels of DPM/ng DNA were 70.0  $\pm$  54.8 and 32.4  $\pm$  15.6, respectively ( $P = 0.040$ ); for Sat2 by the MethyLight assay, values were 42.2%  $\pm$  55.1% and 117.9%  $\pm$  88.8% ( $P < 0.0001$ ); and for LINE1, 48.6  $\pm$  14.8 and 71.7  $\pm$  1.4% ( $P < 0.0001$ ), respectively (Table 1).

For the methyl acceptance assay, in 28 of 37 paired samples (75.7%), methylation in tumor tissues was lower than that in adjacent non-tumor tissues. For Sat2 and LINE1 analysis, in 31 (83.8%) and 32 (86.5%) subjects, levels were lower in tumor than in adjacent non-tumor tissues, respectively.

Plasma levels of AFB<sub>1</sub>-Alb adducts had been measured previously in bloods collected at the time of surgery<sup>[22]</sup>. As hypothesized, plasma levels of AFB<sub>1</sub>-Alb adducts were statistically significantly inversely correlated with methylation levels of LINE1 ( $r = -0.36$ ,  $P = 0.034$ ) (Table 2 and Figure 1). Plasma levels of AFB<sub>1</sub>-Alb adducts were also inversely correlated with tumor methylation levels measured by Sat2, but not statistically significantly ( $r = -0.30$ ,  $P = 0.082$ , Table 2). Since higher values in the methyl acceptance assay indicate hypomethylation, the correlation between adducts and methylation in this assay is also in the correct direction but not significant ( $r = 0.18$ ,  $P = 0.286$ , Table 2).

The associations of HBV and HCV infection sta-



**Figure 1** The correlation between aflatoxin B<sub>1</sub>-Alb levels in plasma (aflatoxin B<sub>1</sub>/μg) and long interspersed nucleotide element-1 methylation (%) in tumor tissue. AFB<sub>1</sub>: Aflatoxin B<sub>1</sub>; LINE1: Long interspersed nucleotide element-1.

tus, cirrhosis status and promoter hypermethylation of *p16<sup>Ink4A</sup>* and *Hm1* with global hypomethylation in tumor tissue are given in Table 3. No statistically significant correlations were found, except for LINE1 and being positive for both HBV and HCV infection. However, only one subject was negative for both HBV and HCV so this result is likely to be spurious.

## DISCUSSION

Hypomethylation was observed in tumor compared to adjacent non-tumor tissues using three different assays that measure global methylation or methylation in two repetitive elements. The level of [<sup>3</sup>H]-methyl acceptance of HCC tumor DNAs was statistically significantly higher compared to that of adjacent non-tumor DNAs ( $P < 0.040$ ), indicating significantly lower methylation. This is the first study to report that global hypomethylation contributes to hepatocarcinogenesis using the [<sup>3</sup>H]-methyl acceptance assay. Global loss of methylation in cancer may lead to alterations in the expression of proto oncogenes critical to carcinogenesis and facilitate chromosomal instability<sup>[28]</sup>.

Repetitive DNA elements are normally heavily methylated and a previous study showed a correlation between Alu, Sat2 and LINE1 methylation by MethyLight and 5<sup>m</sup>C content in normal and tumor samples<sup>[20]</sup>, indicating the usefulness of these assays as surrogate measures of genomic methylation levels. In this study, tumor methylation was statistically significantly lower than in paired adjacent non-tumor tissue for Sat2 ( $P < 0.0001$ ) by the MethyLight assay and for LINE1 ( $P < 0.0001$ ) by pyrosequencing, consistent with the data from the methyl acceptance assay and as reported previously<sup>[14,27,28]</sup>.

A previous study found that three repetitive DNA elements, Sat2, Alu and LINE1, showed discordance in timing of hypomethylation along the multistep pathway

in hepatocarcinogenesis from normal liver to HCC; Sat2 hypomethylation occurred at the chronic hepatitis stage<sup>[29]</sup>. Hypomethylation also differed according to geographic location of the subjects and their hepatitis infection status; mean LINE1 methylation in tumor samples was lower in hepatitis-positive cases than in hepatitis-negative cases<sup>[30]</sup>. These findings suggest that HBV or HCV infection can influence global DNA hypomethylation status. This may be partially explained by the fact that the HBV X protein can induce altered DNA methyltransferase activity, hypermethylation of specific CpG islands and global hypomethylation<sup>[30,31]</sup>. In the present study, no associations between DNA global hypomethylation and HBV and HCV infection were observed (Table 3). However, only one case was negative for markers of infection for either HBV or HCV, limiting our ability to investigate the role of infection on methylation levels.

Exposure to AFB<sub>1</sub> is one of the major risk factors for the development of HCC. In our previous studies, we found a strong relationship between AFB<sub>1</sub> exposure and promoter hypermethylation in tumor suppressor and other cancer-related genes, including *RASSF1A*<sup>[32]</sup>, *p16<sup>Ink4A</sup>*<sup>[22,32]</sup> and *MGMT*<sup>[33]</sup> in tumor tissues and plasma DNA of HCC patients. AFB<sub>1</sub> may bind preferentially to methylated CpG sites and/or specific structures in chromatin, inducing damage to DNA and histones<sup>[33]</sup> that may impact on methylation. Several other environmental exposures have been associated with epigenetic changes. Increasing air levels of benzene, a chemical carcinogen, was associated with a significant reduction in LINE1 and Alu1 methylation in white blood cells<sup>[34]</sup>. LINE1 DNA methylation is also inversely associated with lead exposure in humans<sup>[35]</sup>. Even although the mechanisms are still not clear, these data suggest that exposure to some chemical carcinogens may cause changes in global methylation status. In the present study, plasma levels of

**Table 3** Methylation levels in tumor tissues and clinical characteristic and gene-specific methylation in tumor tissues

Variable	<i>n</i>	LINE1 (%) mean ± SD	<i>P</i> value	Sat2 (%) mean ± SD	<i>P</i> value	Methyl acceptance (DPM/ng) mean ± SD	<i>P</i> value
HBsAg							
Negative	5	41.0 (16.6)	0.245	22.1 (19.2)	0.905	67.2 (37.6)	0.607
Positive	26	47.7 (13.6)		31.3 (34.5)		81.3 (58.3)	
AntiHCV							
Negative	18	49.6 (14.2)	0.449	27.6 (22.6)	0.737	72.1 (49.7)	0.759
Positive	10	45.2 (14.3)		38.0 (47.7)		86.5 (71.3)	
HBsAg and AntiHCV							
Both negative	1	24.9	0.047	5.7	0.323	64.5	0.877
Either one positive	31	47.2 (13.5)		31.1 (32.2)		78.4 (55.5)	
Cirrhosis							
No	17	44.8 (15.2)	0.388	28.1 (23.8)	0.791	78.6 (52.4)	0.927
Yes	13	48.9 (13.2)		32.5 (43.1)		82.3 (61.9)	
<i>p16<sup>ink4A</sup></i>							
Unmethylated	15	50.9 (16.7)	0.569	63.4 (80.8)	0.437	62.0 (61.2)	0.155
Methylated	22	47.1 (13.5)		27.8 (21.1)		75.4 (50.8)	
Hint1							
Unmethylated	17	50.9 (14.8)	0.405	51.8 (75.8)	0.951	60.2 (48.9)	0.142
Methylated	20	46.7 (14.8)		34.1 (30.3)		78.3 (59.4)	

HCV: hepatitis V virus; LINE1: Long interspersed nucleotide element-1; Sat2: Satellite 2; DPM: Disintegrations/min.

AFB<sub>1</sub>-Alb adducts were statistically significantly inversely correlated with methylation levels of LINE1, providing additional evidence that carcinogens may alter global methylation. Reactive oxygen species and the resulting DNA damage produced by AFB<sub>1</sub> may reduce binding affinity of methyl-CpG binding protein 2, therefore resulting in epigenetic alterations<sup>[36,37]</sup>.

It is still uncertain whether or not gene-specific promoter hypermethylation and global hypomethylation are independent processes; in HCC, their correlation is still controversial. One recent study demonstrated that global hypomethylation in HCC was associated with gene-specific hypermethylation<sup>[30]</sup>, but another showed variability between individual CpG islands' hypermethylation and repetitive DNA hypomethylation status and concluded that there is no mechanistic link in liver cancer cells<sup>[29]</sup>. We also found no association between promoter hypermethylation in the two specific genes investigated and global hypomethylation in HCC tissue DNAs. In addition, gene-specific hypermethylation and global hypomethylation appear to be independent processes in colon and urothelial cancers<sup>[38,39]</sup>. Further investigations are still needed to validate the relationship between global hypomethylation and gene-specific promoter hypermethylation.

In summary, this is the first study to investigate global hypomethylation, one of the most consistent epigenetic changes in cancer development in HCC, and paired adjacent non-tumor tissues using three different methods: the methyl acceptance assay and analysis of Sat2 and LINE1, two repetitive elements. Consistent hypomethylation in tumor compared to adjacent tissue was found by all three

methods. AFB<sub>1</sub> exposure was also associated with DNA hypomethylation, suggesting that chemical carcinogens may influence epigenetic changes in human tissues.

## COMMENTS

### Background

Epigenetic alterations are involved in cancer development and progression. Promoter CpG island hypermethylation contributes to carcinogenesis by shutting off expression of tumor suppressor and DNA repair genes. Genomic DNA hypomethylation is implicated in carcinogenesis by inducing chromosome instability and loss of imprinting. Genome-wide hypomethylation has been reported in a variety of cancers, including hepatocellular carcinoma (HCC). Hypomethylation of the genome mainly affects the intergenic and intronic regions of DNA, particularly repeat sequences and transposable elements. Analysis of methylation levels of Satellite 2 (Sat2) and long interspersed nucleotide element-1 (LINE1) is frequently used as a measure of global methylation. The correlations between global hypomethylation and hepatitis infection status have been investigated, but the association between hypomethylation and aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) exposure in HCC is still unclear.

### Research frontiers

Genomic DNA hypomethylation is a common finding in human cancers. Global DNA hypomethylation reflected in reduced levels of methylation in repeat regions, occurs in target tissues undergoing carcinogenic differentiation, and could be used as a biomarker of malignant tumors. Environmental factors such as geographic location and hepatitis status have been shown to contribute to hepatocarcinogenesis through global hypomethylation.

### Innovations and breakthroughs

In the present study, the authors first investigated DNA methylation in HCC and paired adjacent non-tumor tissues using the methyl acceptance assay as a measure of global methylation. They also analyzed two repetitive elements, including Sat2 by MethyLight and LINE1 by pyrosequencing. With all three assays, mean methylation levels in tumor tissues were significantly lower than that in adjacent non-tumor tissues. They also first found that AFB<sub>1</sub>-albumin adducts levels were inversely correlated with LINE1 methylation, providing an additional mechanism by which exposure to this dietary carcinogen may influence

hepatocarcinogenesis.

### Applications

This work demonstrated that methyl acceptance assay could be used to accurately detect global hypomethylation in HCC samples. Finding that AFB<sub>1</sub> exposure is correlated with global hypomethylation, as well as hypermethylation in some genes, demonstrates the important role it plays in the development of HCC. This may help to develop new strategies to prevent HCC.

### Terminology

Global hypomethylation is a decrease in the overall genomic 5-methylcytosine content (compared to total cytosines) from approximately 4% in normal tissues to 2%-3% in cancer tissues. This change was first observed in a number of studies in 1983, in lung and colon carcinomas compared to adjacent normal tissue, and in various malignancies compared to various postnatal tissues, demonstrating that overall genomic 5-methylcytosine levels were lower in cancer tissues. This observation has been reproducibly repeated in a wide range of cancers and matched normal tissues using a variety of different techniques.

### Peer review

This manuscript addresses an interesting issue for the initiation and progression of HCC.

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