Hepatic DNA Methylation Is Affected by Hepatocellular Carcinoma Risk in Patients with and without Hepatitis Virus

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Abstract

Objectives: Several studies revealed that the proportion of hepatocellular carcinoma (HCC) without hepatitis virus infection (NBNC-HCC) is increasing. On the other hand, epigenetic alterations are reportedly responsible for HCC development. Here, we identified HCC risk factors that are associated with DNA methylation in the background liver tissue of NBNC-HCC patients. Methods: We performed methylation analysis in 37 pairs of virus-positive and 22 pairs of NBNC-HCC and non-cancerous livers using a HumanMethylation450 BeadChip array. After the selection of differentially methylated CpGs (DM-CpGs) in cancerous and non-cancerous livers, we analyzed DNA methylation of DM-CpGs within the adjacent non-cancerous liver tissue that is affected by specific HCC risk factors. Results: A total of 38,331 CpGs were selected as DM-CpGs using the following criteria: difference of β-value between HCC and non-cancerous liver ≥0.15 and false discovery rate (FDR) q < 1.0E-12. We subsequently selected the DM-CpGs that had methylation differences with the background liver tissue (that has FDR q < 0.35). Among the virus-positive patients, the type of hepatitis virus was mostly associated with differences in methylation within the background liver tissues. However, we found that background methylation patterns were most significantly associated with aging in NBNC-HCC patients. Interestingly, age-related methylation differences in DM-CpGs were also observed in NBNC-HCC tissues. Conclusions: Hepatitis viruses affect the methylation profiles within background liver tissues. However, difference in background methylation was mostly associated with age in NCBC-HCC patients; some age-related methylation events could contribute to emergence of NBNC-HCC in elderly individuals.

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the best known causes of hepatocellular carcinoma (HCC), so far [1, 2]. Therefore, recent development of antiviral therapy was aimed at decreasing the...
incidence of hepatitis virus-related HCCs [3]. However, several studies revealed that the proportion of HCC patients, which are not related to HBV and HCV infections (NBNC-HCC), is increasing especially in elderly patients [4, 5]. It is well documented that life style contributes to NBNC-HCC emergence, such as alcohol intake, increased prevalence of non-alcoholic fatty liver disease (NAFLD), diabetes mellitus and aging [6, 7].

Recent comprehensive analyses revealed that many kinds of genetic and epigenetic alterations were observed in background liver and HCC tissues [8, 9]. Therefore, it is conceivable that hepatocytes with genetic and epigenetic alterations in non-cancerous livers could act as a ‘seed’ for HCC emergence. Previously, we reported that number of methylated tumor suppressor genes was associated with time to HCC emergence in chronic hepatitis C patients [10]. This evidence also supported the idea that the degree of epigenetic alterations in chronic hepatitis should be an HCC development risk [11]. It has also been reported that oxidative DNA damage could induce epigenetic alterations that might be critical for hepatocarcinogenesis [12–14]; the known risks of NBNC-HCC, such as alcohol intake and the presence of NAFLD, could induce oxidative stress in hepatocytes [15]. This, in turn, can induce specific epigenetic alterations that are responsible for hepatocarcinogenesis [16, 17]. In this study, we attempted to identify a known HCC risk factor that is associated with DNA methylation in the background liver of NBNC-HCC patients. More specifically, we selected the differentially methylated CpGs (DM-CpGs) loci in HCC and non-cancerous background liver (DM-CpGs). Subsequently, we analyzed the differences in methylation of DM-CpGs in the context of specific risks for NBNC-HCCs and virus-related HCCs. We identified risk factors that were associated with unique HCC-related methylation events in NBNC-HCC background liver.

Materials and Methods

Patients

We performed comprehensive methylation analysis on 22 pairs of cancerous and non-cancerous livers from the NBNC-HCC patients. Similarly, we analyzed hepatic methylation profiles in 37 pairs of cancerous and non-cancerous livers from the hepatitis virus-positive HCC patients. The NBNC-HCC patients had a median age of 72 years (distribution 61–79); 15 were men and 7 were women. The median body mass index (BMI) was 23.5 (distribution 14.6–32.5). Ten of the 22 patients were diabetic, and 4 had cirrhotic whereas 14 had non-cirrhotic background liver tissues. Three HCCs were well-differentiated and 19 were moderately/poorly differentiated. Eight patients were alcoholics (intake >30 g/day for males and >20 g/day for females), whereas 14 were not. Of the 14 non-alcoholic patients, 7 had ballooning accompanied by steatosis in the background liver, which indicates the presence of non-alcoholic steatohepatitis.

The hepatitis virus-positive HCC patients had a median age of 61 years (distribution 42–75), and 25 were men whereas 14 were women. The median BMI was 23.8 (distribution 18.9–33.5). The diabetic status was not determined for 20 patients; of the other 17 patients, 2 were diabetic. Thirty-one had cirrhotic and 8 had non-cirrhotic background liver. Sixteen HCCs were well-differentiated and 23 were moderately/poorly-differentiated. Sixteen patients were positive for the HBV surface antigen, and 23 were positive for HCV antibody.

Selection of DM-CpGs between Cancerous and Non-Cancerous Liver

For comprehensive methylation analyses, we used the Illumina Infinium HumanMethylation450 BeadChip array (Illumina, Inc., San Diego, Calif., USA). Among the 485,512 probes covering 99% of NCBI reference sequence database genes, we eliminated the probes that contained single-nucleotide polymorphisms or those located on sex chromosomes. Details of the methylation analysis using the HumanMethylation450 BeadChip were published previously [18, 19]. DM-CpG selection was performed if there was a difference in β-value between the cancerous and noncancerous liver ≥0.15 and false discovery rate (FDR) q-value using the Benjamini–Hochberg procedure <1.0E-12. Consequently, 38,331 CpGs that met these criteria were considered DM-CpGs.

DM-CpGs in Background Liver Tissues and Specific HCC Risk Factors

Among the 38,331 DM-CpGs described above, methylation profile differences based on HCC risk factors were also explored in adjacent non-cancerous livers. For this purpose, we further selected DM-CpGs between background livers with and without specific HCC risk factors; we analyzed methylation differences within the liver tissues between HBV surface antigen- and HCV antibody-positive patients in the virus-related HCC group. Similarly, we analyzed methylation differences between cirrhotic and non-cirrhotic, male and female and obese (BMI ≥25) and non-obese (BMI <25) livers. DM-CpGs with methylation differences within the background liver with FDR q-value <0.35 were selected as risk factor-specific CpGs.

Statistics

The Mann–Whitney U test and the FDR control using the Benjamini–Hochberg procedure were applied for multiple comparisons. The Pearson’s chi-square test or the Fisher’s exact test was used to compare categorical variables. Principle component analysis and hierarchal clustering analysis were performed to categorize the tissues based on methylation levels. All statistical analyses were conducted using the program R-3.0.3 (www.r-project.org) and JMP version 9.0 software (SAS Institute Inc., Cary, N.C., USA).
Results

DM-CpGs between Cancerous and Non-Cancerous Liver

As described above, we selected 38,331 DM-CpGs. Of these, 3,052 showed increased methylation in HCC tissues, and 35,279 DM-CpGs had decreased methylation in HCC. We performed principle component analysis using β-values of the top 500 DM-CpGs that were most differentially methylated between HCCs and non-cancerous livers (fig. 1a). The HCC tissues had a more scattered distribution than non-cancerous liver tissues, which indicates heterogeneity of methylation profiles in HCC tissues; methylation was homogeneous in non-cancerous livers compared to that of HCCs. HCC tissues were also clearly differentiated from non-cancerous liver using β-values of the top 500 DM-CpGs by hierarchical clustering analysis (fig. 1b).

Risk Factor-Specific DM-CpGs within the Background Liver of Virus-Positive and NBNC-HCC Patients

We selected the risk factor-specific DM-CpGs within the background liver of both hepatitis virus-positive and NBNC-HCC patients as described in the Materials and Methods. In hepatitis virus-positive HCC group, 11,164 of the 38,331 DM-CpGs (29%) were selected as risk factor-specific DM-CpGs between HBV- and HCV-positive background livers and were considered virus-related DM-CpGs. Similarly, we found that 2,896 DM-CpGs were associated with the presence of cirrhosis (cirrhosis-related DM-CpGs), and 1,164 were sex-related DM-CpGs. However, no DM-CpGs were selected between elderly and non-elderly background liver. On the contrary, within the non-cancerous livers of the NBNC-HCC patients, the risk factor most significantly associated with difference of methylation level were aging, 6,517 of 38,331 DM-CpGs (17%) were selected as age-
related DM-CpGs between elderly and non-elderly background liver, followed by the presence of diabetes mellitus, 47 DM-CpGs were diabetes-related risk factors (diabetes-related DM-CpGs). On the other hand, none of the methylation events on the DM-CpGs were affected by the presence or absence of cirrhosis, difference of sex or BMI. Interestingly, we could not find any DM-CpGs between alcoholic and non-alcoholic background liver.

**Age-Related Methylation within Background Liver Could Affect NBNC-HCC Methylation Patterns**

Among the 6,517 age-related DM-CpGs in background liver, 340 had β-values ≥0.15 between elderly and non-elderly livers. We classified tumors by hierarchical clustering analysis based on the β-values of the top 340 age-related DM-CpGs quantified in HCC tissues. We successfully classified HCCs into 2 subgroups (fig. 2); all HCCs that belonged to cluster 2 emerged from elderly patients (p = 0.0053 by the Fisher’s exact test). Alternatively, other parameters, such as sex, alcohol intake, BMI, presence of DM, cirrhosis and tumor differentiation, did not affect this classification, which indicates that the methylation status of age-related DM-CpGs in NBNC-HCC tissues could be specifically affected by that of the background liver in elderly patients.

**Discussion**

Although the overall prevalence of the at-risk population for HCC is still growing, there is a recent trend showing an increase in proportion of NBNC-HCC patients in Japan [1, 5]. There are several risks for developing NBNC-HCC such as alcohol consumption, aging, presence of diabetes mellitus, obesity and hepatic iron overload [20]; some of these could act in concert and contribute to carcinogenesis.

On the other hand, several reports described epigenetic alterations in HCCs [21–23]; these molecular events could be, at least partially, attributed to the induction of oxidative DNA in the damaged hepatocytes. Previously, we reported that oxidative DNA damage could induce abnormal DNA methylation that is associated with HCC emergence [14]. Based on this background, we examined the difference of methylation profiles in the background liver of the HCC patients in the context of specific risks for HCC emergence that was associated with the oxidative stress.

First, we selected the CpGs that showed methylation differences between cancerous and non-cancerous liver (DM-CpGs) based on the β-value obtained from HumanMethylation450 BeadChip array analysis that reflected the methylation level of CpGs. As previously described,
methylation events in the selected 38,331 DM-CpGs loci should represent the unique pattern of HCC-related DNA methylation alterations. Alternatively, it may be possible that some HCC risks might affect the methylation profile within background liver; some of these background alterations should contribute to hepatocarcinogenesis through the ‘epigenetic pathway’. Then, we attempted to clarify which risks could affect the methylation differences of the DM-CpGs within the background liver. Among the virus-positive patients, the type of hepatitis virus (HBV vs. HCV) was most associated with methylation difference in DM-CpGs. Previously, several reports showed the difference of methylation profiles between HBV- and HCV-related HCCs [24]. We also reported methylation differences within the non-cancerous livers of HBV- and HCV-positive patients [8, 25]. Integration of HBV into the host genome is a possible major mechanism of HCC emergence in HBV-related carcinogenesis [26], but this should not be the case in HCV-related HCCs. Therefore, disturbance of the host genome, but not DNA methylation, might be more important in a subset of HBV- than that of HCV-related carcinogenesis. In addition, integration of HBV that could trigger the DNA methylation at flanking genomic sequences should give rise to the methylation difference between HBV- and HCV-positive livers [27].

Our analysis also revealed that the degree of background fibrosis is associated with methylation difference. In the virus-related HCC group, liver fibrosis was more prominent in HCV- than in HBV-positive patients; this might lead to methylation status differences of DM-CpGs between cirrhotic and non-cirrhotic livers [28]. Alternatively, the duration of hepatitis, which should be related fibrosis progression, could affect DM-CpG loci methylation within the background liver. We also found that DM-CpG methylation patterns in background liver were most significantly associated with aging in NBNC patients, followed by the presence or the absence of diabetes mellitus, whereas fibrosis, alcohol intake and degree of BMI did not affect background methylation difference. Previous studies suggested that fibrosis and alcohol intake could affect DNA methylation of the liver tissues [29, 30]. Due to obesity as well as alcohol intake, NAFLD could increase oxidative stress and DNA damage in hepatocytes; therefore, it is conceivable that both NAFLD and alcohol intake might contribute to hepatocarcinogenesis via the same epigenetic pathway, which would lead to a lack of difference in the methylation profile within the background liver tissues, regardless of the fibrosis stage.

We found a large number of DM-CpGs between elderly and non-elderly livers in NBNC group. Therefore, aging may affect the DNA methylation in the background liver, which has also been previously reported. In addition, these age-related DM-CpGs methylation in NBNC patients were also observed in HCC tissues. This evidence also supports the idea that methylation events that emerge during the aging process might play a role in HCC development [31], and clonal expansion of hepatocytes with age-related methylation could take place. The specific age-related methylation events that could drive carcinogenesis in virus-negative patients are currently under investigation.

In this report, we showed that the type of hepatitis virus affected the methylation profile in the background liver of HBV- and HCV-related HCC patients. However, methylation difference within the background liver was most prominent between elderly and non-elderly NCBC-HCC patients; some of these methylation events could contribute to emergence of NBNC-HCC. Further studies are necessary to elucidate the role of age-related epigenetic alteration on NBNC-HCC emergence.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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