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ORIGINAL ARTICLE

Basic Study

DNA methylation of angiotensin Ⅱ receptor gene in nonalcoholic steatohepatitis-related liver fibrosis

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Author contributions: Asada K performed the majority of experiments and analyzed the data; Aihara Y and Takaya H performed the molecular investigations; Noguchi R, Namisaki T, Moriya K, Uejima M, Kitade M, Mashitani T, Takeda K, Kawaratani H, Okura Y, Kaji K, Douhara A, Sawada Y, Nishimura N and Seki K participated in treatment of animals; Mitoro A, Yamao J and Yoshiji H designed and coordinated the research; Asada K, Kaji K and Yoshiji H wrote the paper.

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Abstract

AIM

To clarify whether *Agtr1a* methylation is involved in the development of nonalcoholic steatohepatitis (NASH)-related liver fibrosis in adult rats.

METHODS

A choline-deficient amino acid (CDAA) diet model was employed for methylation analysis of NASH-related liver fibrosis. *Agtr1a* methylation levels were measured in the livers of CDAA- and control choline-sufficient amino acid (CSAA)-fed rats for 8 and 12 wk using quantitative methylation-specific PCR. Hepatic stellate cells (HSCs) were isolated by collagenase digestion of the liver, followed by centrifugation of the crude cell suspension through a density gradient. *Agtr1a* methylation and its gene expression were also analyzed during the activation of HSCs.

RESULTS

The mean levels of *Agtr1a* methylation in the livers of CDAA-fed rats (11.5% and 18.6% at 8 and 12 wk, respectively) tended to be higher (P = 0.06 and 0.09, respectively) than those in the livers of CSAA-fed rats (2.1% and 5.3% at 8 and 12 wk, respectively). *Agtr1a* was not methylated at all in quiescent HSCs, but was clearly methylated in activated HSCs (13.8%, $P < 0.01$). Interestingly, although *Agtr1a* was hypermethylated, the *Agtr1a* mRNA level increased up to 2.2-fold ($P < 0.05$) in activated HSCs compared with that in quiescent HSCs, suggesting that *Agtr1a* methylation did not silence its expression but instead had the potential to upregulate its expression. These findings indicate that *Agtr1a* methylation and its upregulation of gene expression are associated with the development of NASH-related liver fibrosis.

CONCLUSION

This is the first study to show that DNA methylation is potentially involved in the regulation of a renin-angiotensin system-related gene expression during liver fibrosis.

Key words: Epigenetics; DNA methylation; Angiotensin Ⅱ receptor; Liver fibrosis; Nonalcoholic steatohepatitis

Core tip: We report the first study to show that *Agtr1a* methylation occurred during the development of nonalcoholic steatohepatitis-related liver fibrosis. Interestingly, *Agtr1a* gene expression was upregulated during liver fibrosis, although *Agtr1a* was methylated. This study demonstrates for the first time that renin-angiotensin system-related gene expression is regulated by DNA methylation during liver fibrosis. This finding raises expectations about the therapeutic application of demethylating agents for the treatment of liver fibrosis.

Asada K, Aihara Y, Takaya H, Noguchi R, Namisaki T, Moriya K, Uejima M, Kitade M, Mashitani T, Takeda K, Kawaratani H, Okura Y, Kaji K, Douhara A, Sawada Y, Nishimura N, Seki K, Mitoro A, Yamao J, Yoshiji H. DNA methylation of angiotensin Ⅱ receptor gene in nonalcoholic steatohepatitis-related liver fibrosis. *World J Hepatol* 2016; 8(28): 1194-1199 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i28/1194.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i28.1194

INTRODUCTION

Liver fibrosis is a characteristic feature of chronic liver disease regardless of the etiology. Cirrhosis is the terminal condition of chronic liver diseases, and hepatic failure due to liver cirrhosis is caused by progressive fibrosis that ultimately results in nodular regeneration with loss of function[1-3]. Considering that hepatocellular carcinoma (HCC) also develops from liver fibrosis, it is necessary to investigate the molecular mechanisms underlying liver fibrosis development to reduce the morbidity and mortality of chronic liver disease.

The renin-angiotensin system (RAS) is continually activated in patients with chronic liver diseases, such as cirrhosis[4]. Angiotensin Ⅱ (AT-Ⅱ), an octapeptide produced mainly *via* the enzymatic cleavage of angiotensin I by angiotensin I-converting enzyme, reportedly plays an important role in chronic liver disease progression. AT-Ⅱ activates a series of signal transduction pathways in activated hepatic stellate cells (HSCs) by binding to the AT-Ⅱ type 1 receptor (AT1-R)[5]. We previously reported that AT1-R blockers significantly attenuate experimental liver fibrosis development with the suppression of activated HSC proliferation[6-8]. However, the molecular mechanisms regulating RAS-related gene expression remain unelucidated.

Epigenetic alterations, including DNA methylation, are involved in the progression of liver fibrosis and HCC in human and animal studies[9-11]. Recently, Chen *et al*[12] reported that RAS-related genes, especially *Agtr1a* encoding rat AT1-R, are methylated in rats born to mothers fed a methyl donor-deficient diet during gestation and lactation. They showed that *Agtr1a* methylation can be a surrogate marker to predict susceptibility in developing nonalcoholic fatty liver disease (NAFLD) later in life. However, it is unclear whether *Agtr1a* methylation is associated with the development of nonalcoholic steatohepatitis (NASH)-related liver fibrosis.

Here we employed choline-deficient amino acid (CDAA)-fed rats to evaluate the importance of *Agtr1a* methylation in the development of NASH-related liver fibrosis. Our results demonstrate that *Agtr1a* methylation is potentially associated with liver fibrosis development and HSC activation.

MATERIALS AND METHODS

Animal model of liver disease

Six-week-old male Fisher 344 rats (CLEA Japan, Inc., Osaka, Japan) were housed in a room under a controlled temperature and a 12/12-h light-dark cycle. The animals were divided into the following four experimental groups: (1) choline-sufficient amino acid diet (CSAA) for 8 wk (n = 4); (2) CSAA for 12 wk (n = 11); (3) CDAA for 8 wk (n = 10); and (4) CDAA for 12 wk (n = 12). Initially, sample sizes for group (1)-(5) were 5, 12, 10, and 12, respectively, but two animals (one for CSAA-diet for 8 wk and the other for CSAA-diet for 12 wk) were dropped out because of entry in another experiment. All animal procedures were performed in accordance with standard protocols and following the standard recommendations for the appropriate care and use of laboratory animals. This study was approved by the animal experiment ethical committee at the Nara Medical University (protocol number: 9354).

Isolation and activation of HSCs

HSCs were isolated by the collagenase digestion of the liver of a 6-week-old male Fisher 344 rat using a

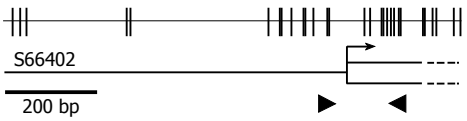

Figure 1 *Agtr1a* genomic structure. Each vertical tick on the top line shows an individual CpG site. GenBank accession number is listed at the left end on the bottom line. Open box shows exon 1, and dashed lines show the ambiguous boundary region of exon 1. Quantitative real-time methylation-specific PCR was performed in the region marked with closed arrowheads.

perfusion system, followed by the centrifugation of the crude cell suspension through a density gradient, as described previously[13]. Genomic DNA and total RNA were isolated from freshly isolated HSCs in a quiescent state. Thereafter, HSCs were activated in a culture on a plastic dish for 5 d.

Genomic DNA isolation, sodium bisulfite modification, and quantitative real-time methylation-specific PCR

Genomic DNA was isolated using a DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). Fully methylated control DNA was prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA), and completely unmethylated control DNA was purchased from EpigenDx (Hopkinton, MA). Bisulfite modification was performed using an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). *Agtr1a* genomic structure is illustrated in Figure 1. An aliquot of 1 μL was used for quantitative real-time methylation-specific PCR (qMSP) with primers specific to a methylated sequence of *Agtr1a* (forward 5′-GGT TGG AAT TTG TAG AGT AGC GAC-3′, reverse 5′-CAA CGC TAA TAC CGA CCT CG-3′) and to a B2 repeat sequence, regardless of the methylation status, as demonstrated in a previous report[14].

qMSP was performed by real-time PCR using a Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) and a StepOnePlus™ Real-Time PCR® (Thermo Fisher Scientific, Waltham, MA). The methylation level was calculated as the methylation percentage obtained as follows: {[number of DNA molecules methylated at a target CpG island (CGI) in a sample]/(number of B2 repeats in the sample)}/[(number of DNA molecules methylated at the target CGI in completely methylated control DNA)/(number of B2 repeats in the completely methylated control DNA)] × 100, as described previously[15].

Quantitative real-time reverse transcription PCR

Total RNA was extracted using an RNeasy® Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg of total RNA using a High Capacity RNA to cDNA Master Mix (Thermo Fisher Scientific, Waltham, MA). *Agtr1a* mRNA level was measured by quantitative PCR using the StepOnePlus™ Real-Time PCR® (Thermo Fisher Scientific, Waltham, MA). Primer sequences for *Agtr1a* and for *Ppia* were reported previously[14,16]. The number of *Agtr1a* cDNA molecules was normalized to that of *Ppia* cDNA molecules.

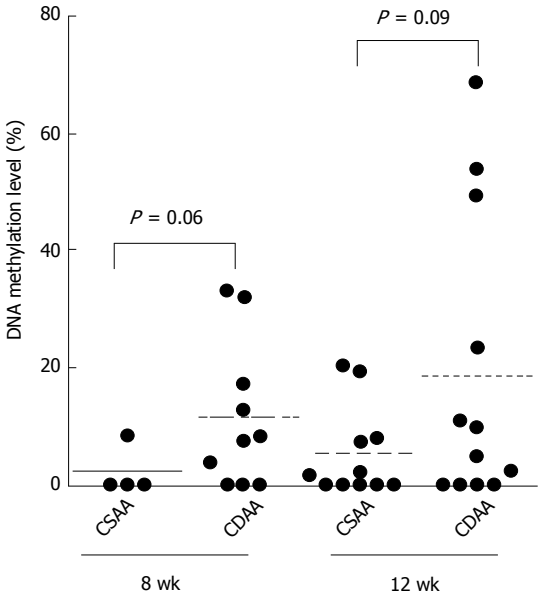

Figure 2 Levels of *Agtr1a* methylation in the livers of control choline-sufficient amino acid - and choline-deficient amino acid - fed rats. The livers of choline-deficient amino acid (CDAA) - fed rats show higher *Agtr1a* methylation than that shown by the livers of choline-sufficient amino acid (CSAA) - fed rats at 8 (mean, 11.5% and 2.1%, $P = 0.06$) and 12 wk (mean, 18.6% and 5.3%, $P = 0.09$), respectively.

Statistical analysis

The difference in mean methylation levels was analyzed using Welch's *t*-test. The results were considered significant with a P value of < 0.05.

RESULTS

Agtr1a methylation in the livers of CDAA-fed rats and activated HSCs

To evaluate the status of *Agtr1a* methylation in the whole liver, we performed qMSP using the liver samples of CSAA- and CDAA-fed rats after the two feeding periods, 8 and 12 wk. The mean levels of *Agtr1a* methylation in the livers of CDAA-fed rats were 11.5% and 18.6% at 8 and 12 wk, respectively, whereas those in the livers of CSAA-fed rats were 2.1% and 5.3% at 8 and 12 wk, respectively. These findings suggested that the levels of *Agtr1a* methylation in the livers of CDAA-fed rats tended to be higher than those in the livers of CSAA-fed rats at 8 and 12 wk ($P = 0.06$ and 0.09, respectively; Figure 2).

Next, we evaluated the level of *Agtr1a* methylation during HSC activation *in vitro*. We found that *Agtr1a* methylation was not detected at all in quiescent HSCs, but was clearly observed in activated HSCs (13.8%, $P < 0.01$; Figure 3). Taken together with the *in vivo* results, our findings indicate that *Agtr1a* is hypermethylated in accordance with the development of NASH-related liver fibrosis.

Agtr1a expression in activated HSCs, and its association with methylation

To address the contribution of *Agtr1a* methylation to its

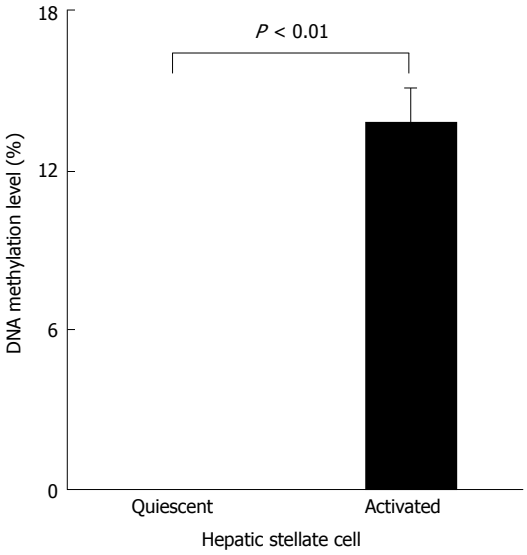

Figure 3 Levels of *Agtr1a* methylation in the quiescent and activated hepatic stellate cells. *Agtr1a* is not methylated at all in quiescent hepatic stellate cells (HSCs) but hypermethylated (13.8%, $P < 0.01$) in activated HSCs. Data are presented as the mean ± SE.

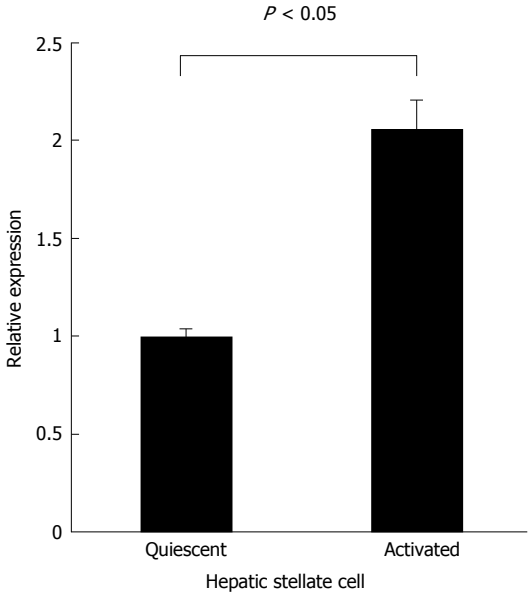

Figure 4 Relative *Agtr1a* expression normalized to *Ppia* in quiescent and activated hepatic stellate cells. Activated hepatic stellate cells (HSCs) show 2.2-fold higher ($P < 0.05$) *Agtr1a* expression than that shown by quiescent HSCs. Data are presented as the mean ± SE.

gene expression, we performed quantitative real-time reverse transcription PCR using quiescent and activated HSCs. *Agtr1a* expression was observed in quiescent HSCs in which *Agtr1a* was unmethylated. Unexpectedly, *Agtr1a* expression increased up to 2.2-fold ($P < 0.05$) in the activated HSCs compared with that in quiescent HSCs, although *Agtr1a* was methylated (Figure 4). Interestingly, in contrast to the general relationship between promoter CGIs and gene expression, *Agtr1a* methylation did not silence its expression but instead had the potential to upregulate its expression.

DISCUSSION

In this study, we found that *Agtr1a* methylation occurred during the development of NASH-related liver fibrosis. *Agtr1a*, which encodes rat AT1-R, the receptor for AT-Ⅱ, is an important factor in liver fibrosis development[17,18]. Our previous reports demonstrated that both *AT-Ⅱ* and *AT1-R* gene expressions were upregulated during fibrosis development in rat liver, and the blockage of AT-Ⅱ/AT1-R signaling could attenuate liver fibrosis[6-8]. Considering that *Agtr1a* methylation upregulates its gene expression, *Agtr1a* demethylation can suppress liver fibrosis.

Agtr1a methylation was first demonstrated in the liver of rats born to mothers fed a methyl donor-deficient diet during gestation and lactation, and it was reported that rat pups with *Agtr1a* methylation have a high risk of developing NAFLD[12]. Epigenetics derived from mother-pup interaction is a prominent research field, and epigenetic susceptibility to phenotypes and diseases, such as yellow coat color, stress response, and breast cancer in offspring, has been identified[19-21]. However, few studies have focused on whether these epigenetic changes responsible for susceptibility to particular diseases occur when the diseases actually develop in adults. Here we found that *Agtr1a* methylation, associated with susceptibility to NAFLD in pups, occurs in liver fibrosis development in adult NASH model rats.

As an experimental NASH model, we employed the CDAA model in this study. In the CDAA model, liver fibrosis develops at 8 wk and severely progresses at 12 wk[22,23]. This model has an advantage of histological progression of liver fibrosis, which is very similar to human NASH. However, there are critical disadvantages of this model. For examples, obesity, glucose intolerance, and insulin resistance, which are common features in human NASH, are not observed in this model. It remains to be elucidated whether *Agtr1a* methylation is induced in other experimental NASH models.

In CDAA model, *Agtr1a* methylation in the livers of CDAA-fed rats tended to be higher than that in the livers of CSAA-fed rats, but it was not statistically significant. We consider that methylation levels are highly variable in each diet group and the difference between CDAA- and CSAA-fed rats appears to be small. This variability depends on individual differences in rats and tissue heterogeneity in each sample, but both of them are hardly avoided. On the other hand, in HSC analysis, *Agtr1a* methylation and upregulation was clearly observed. Even in the CDAA model, it would be better to isolate HSC from the livers of CDAA-fed rats to obtain clear methylation changes.

Agtr1a hypermethylation was associated with *Agtr1a* upregulation. As for the promoter CGI, hypermethylation is generally considered to be strongly associated with gene silencing[24]. On the other hand, in the case of a CGI at the gene body, hypermethylation occasionally contributes to overexpression[25]. In the *Agtr1a* gene, 5′-CGI was not located at the promoter region but was just downstream of the transcription initiation site (Figure 1),

which might contribute to gene overexpression. It is hoped that the mechanism by which gene body methylation induces overexpression can be demonstrated.

In conclusion, this study demonstrates for the first time that RAS-related gene expression is regulated by DNA methylation during liver fibrosis. This finding raises expectations about the therapeutic application of demethylating agents for the treatment of liver fibrosis.

COMMENTS

Background

The renin-angiotensin system (RAS) plays a crucial role in the development of liver fibrosis. Among the RAS-related genes, the methylation of *Agtr1a*, the rat Angiotensin Ⅱ type 1 receptor gene, is a potential risk marker for the development of nonalcoholic fatty liver disease in rat pups. However, it remains to be elucidated whether *Agtr1a* methylation occurs in liver fibrosis development in adult rats with nonalcoholic steatohepatitis (NASH).

Research frontiers

Epigenetics derived from mother-pup interaction is a prominent research field. However, few studies have focused on whether these epigenetic changes responsible for susceptibility to particular diseases occur when the diseases actually develop in adults.

Innovations and breakthroughs

This study demonstrates for the first time that the expression of *Agtr1a*, a RAS-related gene, is regulated by DNA methylation during liver fibrosis.

Applications

The authors finding raises expectations about the therapeutic application of demethylating agents for the treatment of liver fibrosis.

Terminology

Epigenetics refers to heritable marks regulating tissue-specific gene expression without changes in the DNA sequence. Prominent epigenetic marks consist of DNA methylation and histone modifications. Aberrant epigenetic changes are involved in various diseases, including cancer.

Peer-review

This manuscript addresses the role of DNA methylation of angiotensin II receptor in fibrosis development in a rat model of NASH. The study is original and well designed.

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