Reactive Oxygen Species Induce Epigenetic Instability through the Formation of 8-Hydroxydeoxyguanosine in Human Hepatocarcinogenesis

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Key Words

Chronic hepatitis C · Hepatocellular carcinoma · Tumor suppressor genes · Chromatin immunoprecipitation

Abstract

Chronic hepatitis C (CHC) triggers oxidative stress and contributes to the emergence of hepatocellular carcinoma (HCC). We previously reported that tumor suppressor gene (TSG) methylation is a critical factor during the early stages of hepatocarcinogenesis. In this study, we clarify the association between oxidative stress and epigenetic alterations during hepatocarcinogenesis. We examined DNA oxidation and methylation profiles in 128 liver biopsy samples from CHC patients. The DNA oxidation and methylated TSG numbers were quantified using immunohistochemical analysis of 8-hydroxydeoxyguanosine (8-OHdG) and quantitative PCR for 11 TSGs, respectively. The quantitative chromatin immunoprecipitation-PCR (ChIP-qPCR) assay in HepG2 and fetal liver Hc cells treated with H₂O₂ was used to quantify trimethyl-H3K4, acetylated-H4K16 (an active chromatin marker), trimethyl-H3K27 (a repressive chromatin marker) and 8-OHdG. We analyzed 30 promoters of 25 different

TSGs by qPCR. The high levels of 8-OHdG was the only variable that was significantly associated with the increased number of methylated TSGs in CHC ($p < 0.0001$). The ChIP-qPCR revealed that after H₂O₂ treatment of the cell lines, the 8-OHdG-bound promoters showed a modification from an active chromatin (trimethyl-H3K4 and acetylated-H4K16 dominant) to a repressive chromatin (trimethyl-H3K27 dominant) status. We conclude that oxidative stress alters the chromatin status, which leads to abnormal methylation of TSGs, and contributes to hepatocarcinogenesis in CHC patients.

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Introduction

It has been reported that chronic inflammation is a critical factor in the development of cancers such as hepatocellular carcinoma (HCC). Additionally, oxidative stress due to increased production of reactive oxygen species (ROS) plays a pivotal role in carcinogenesis by inducing DNA damage [1, 2]. These DNA alterations, which activate oncogenes and inactivate tumor suppressor

genes (TSGs), could contribute to the development of cancer in the oxidative stress-affected organs [3, 4].

A well-known marker of oxidative DNA damage induced by ROS is 8-hydroxydeoxyguanosine (8-OHdG). A high level of 8-OHdG could be considered as a risk factor in several types of cancer. For example, a higher accumulation of 8-OHdG has been reported in breast, renal and gastric tumor tissues, when compared to adjacent or normal tissues [5–7]. In addition, the cumulative incidence rate of HCC in patients with high 8-OHdG levels in the liver was significantly greater than that in patients with low 8-OHdG levels [8]. Generally, the genotoxic effect of ROS could increase the carcinogenic potential through the induction of base modifications. The formation of 8-OHdG is known to induce G>T/C>A transversions; unrepaired 8-OHdG causes transversion mutations due to its ability to pair with adenine as well as cytosine bases [9]. However, the recently published whole genome analysis suggests that the mutational spectrum of this type of tumor is heterogeneous [10]. Therefore, it is conceivable that an alternative mechanism for the modulation of cancer-related genes, such as the inactivation of TSGs through epigenetic pathways, might be induced by an increase in 8-OHdG levels.

Previously, we have reported that a number of TSGs, which were abnormally hypermethylated in chronic hepatitis C (CHC) liver tissues, were strongly and independently associated with an early onset of HCC in patients without any history of HCCs [11]. However, the mechanism responsible for the induction of abnormal methylation of the TSG promoters is still ambiguous. In this study, we addressed this important issue by focusing on the association between the degree of oxidative stress and hypermethylation of TSGs, which is a critical factor in the early stages of human hepatocarcinogenesis. Here, we clearly demonstrate that the generation of ROS and 8-OHdG could play a critical role in the abnormal methylation of TSGs, which in turn should play an important role in the early stages of HCV-related human hepatocarcinogenesis.

Materials and Methods

Subjects

For analyzing the relationship between the accumulation of 8-OHdG and hypermethylation of TSGs in CHC, we used the biopsy specimens from 126 CHC subjects, who had no prior history of HCC. The detailed information describing the patient cohort has been described previously [11]. The median age of the patients (25–75%) was 57 (46–64), and the cohort included 85 male and 43

female subjects. The liver fibrosis stage (F-stage) of each biopsy specimen was determined by the METAVIR scoring system. Among the subjects, 6 cases were scored as F0, 40 were F1, 37 were F2, 23 were F3 and 22 were F4. Informed consent was obtained from each patient and the study was approved by the institutional review boards of the involved institutions.

Immunohistochemical Analyses of 8-OHdG

We used an archive of liver biopsy specimens, which were fixed in buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and Masson's trichrome for assessment of morphological changes and the stage of fibrosis. Immunohistochemistry (IHC) staining of 8-OHdG was performed using the avidin-biotin complex method. A mouse monoclonal antibody against 8-OHdG (Japan Institute of the Control of Aging, NIKKEN SEIL Co. Ltd., Tokyo, Japan) was used as described previously [12]. A semiquantitative estimation of the degree of 8-OHdG formation was done by counting the number of stained hepatocyte nuclei. The 8-OHdG levels were classified as strong (50% or more of total hepatocytes; fig. 1a), moderate (10–49%; fig. 1b) and weak (9% or less; fig. 1c). Perls' Prussian blue staining was performed to evaluate the levels of iron deposits. The histologic quantification of hepatic iron was based on the number of hepatocytes showing positive Perls' Prussian blue staining: strong (25% or more of total hepatocytes; fig. 1d), moderate (5–24%; fig. 1e), and weak (4% or less; fig. 1f). For both IHC and Perls' Prussian blue staining, ten visual fields from different areas of the liver were used for the evaluation, and 100 nuclei of hepatocytes were counted for each visual field.

Analyses of Abnormal TSG Methylation in Hepatitis Tissue

The methylation status of the promoters of the 11 TSGs was analyzed in the CHC biopsy specimens. Methylation events of 11 TSGs, *HIC1*, *GSTP1*, *SOCS1*, *RASSF1*, *CDKN2A*, *APC*, *RUNX3*, *PRDM2*, *CASP8*, *CACNA1G* and *PTGS2* genes were determined. These TSGs were selected because of a unique profile of abnormal methylation in early HCC [11]. For determination of abnormal methylation, we performed quantitative MethyLight assays using the StepOne™ real-time detection system (Applied Biosystems, Foster City, Calif., USA). The methylation status of 8 TSGs (*HIC1*, *GSTP1*, *SOCS1*, *RASSF1*, *CDKN2A*, *APC*, *RUNX3* and *PRDM2*) in CHC specimens were reported previously [11]; PCR primers and probes for these TSGs have already been described. We further examined 3 additional TSGs, *CASP8*, *CACNA1G* and *PTGS2* in this study. The sequences of PCR primers and Taq-Man probes of these 3 TSGs are as follows: the *CASP8*-forward primer, 5'-AAGTATGTTTAGCGTTCGGGTTTT-3', *CASP8*-reverse primer, 5'-ATACCCAATTTCCAACCATTC-3', and *CASP8*-probe, FAM-TTGTACGTTTATGAATTGT-MGB; the *CACNA1G*-forward primer, 5'-TTTTTTCGTTTCGCGTTT-3', *CACNA1G*-reverse primer, 5'-CTCGAAACGACTTCGCCG-3', and *CACNA1G*-probe, FAM-AAATAACGCCGAATCCGACAACCGA-MGB; the *PTGS2*-forward primer, 5'-CGGAAGCGTTCGGGTAAAG-3', *PTGS2*-reverse primer, 5'-AATTCCACCGCCCCAAAC-3', and *PTGS2*-probe, FAM-TTTCGCCCAAATATCTTTTCTTCTTCGCA-MGB. The PCR was performed following the manufacturer's protocol using Taq-Man® Fast universal PCR Master Mix (Applied Biosystems), as reported previously [11].

Fig. 1. Immunohistochemical staining of 8-OHdG (**a–c**) and iron staining using Perls' Prussian blue (**d–f**). Among 128 biopsy specimens, 34 showed positive staining of 50% or more of the total hepatocyte nuclei and were classified as strong (2+) staining (**a**); similarly, 30 were classified as moderate (1+) staining (10–49% of total

nuclei; **b**) and 61 were weak (±) staining (less than 10%; **c**). The classification of the specimens based on the hepatic iron deposits were as follows: 10 were classified as strong (2+) deposits (25% or more of total hepatocyte; **d**), 40 were moderate (1+) deposits (5–24%; **e**) and 75 were shown as weak (±) deposits (less than 5%; **f**).

Reagents and Cell Lines for Chromatin Immunoprecipitation

The human HCC cell line, HepG2, was obtained from American Type Culture Collection (Rockville, Md., USA) and human fetal liver Hc cells was purchased from Applied Cell Biology Research Institute (Kirkland, Wash., USA). HepG2 and Hc cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified atmosphere with 5% CO₂ at 37°C. Antibodies for chromatin immunoprecipitation (ChIP) were as follows: 8-OHdG (Japan Institute of the Control of Aging, NIKKEN SEIL Co. Ltd.), acetylated-H4K16 (AcK16H4; Millipore, Billerica, Mass., USA), trimethyl-H3K4 (3MeK4H3), trimethyl-H3K27 (3MeK27H3), pan-histone H3 (H3; Wako Pure Chemical Industries Ltd., Osaka, Japan), and rabbit IgG (Genetein Co. Ltd., Tokyo, Japan).

Treatment of HCC Cell Lines with Hydrogen Peroxide, and ChIP before and after Treatment with Hydrogen Peroxide

HepG2 and Hc cells were treated with hydrogen peroxide (H₂O₂), and ChIP was performed before and after treatment using antibodies against AcK16H4 and 3MeK4H3 (an active histone marker), 3MeK27H3 (a repressive histone marker), 8-OHdG (a DNA damage marker), H3 (ChIP-positive control) and rabbit IgG (ChIP-negative control). In 10-cm culture dishes, 3 × 10⁶ of the cells were seeded 24 h prior to the experiments. HepG2 cells were treated with 250 μM H₂O₂ for 1 h and Hc cells were treated with 50 μM of H₂O₂ for 1 h, respectively. The cells were harvested with trypsin, washed twice and suspended in 0.5 ml of PBS. For the cross-linking of histone and DNA, 13.5 μl of 36.6% (w/v) of formaldehyde was added, and cells were incubated for 8 min at room temperature. In order to stop the crosslink reaction, 57 μl of 1.25 M glycine was subsequently added to the cells; cells were incubated for 5 min at room

temperature and washed with cold PBS. For the ChIP reaction for histone, we used Auto ChIP kit and the SX-8G IP-Star Automated System (Diagenode Inc., Denville, N.J., USA). Chromatin was sheared to a length of 400–800 bp using sonication instruments (Bioruptor®). For the 8-OHdG ChIP reaction, we used the Auto MeDIP kit (Diagenode Inc.). The chromatin was incubated with antibodies for 10 h, antibody precipitated and eluted from the magnetic beads following the manufacturer's protocol (Diagenode Inc.).

Quantitative ChIP-PCR Analyses of the Promoters of TSGs

Quantitative ChIP-PCR (ChIP-qPCR) of 30 gene promoters of 25 different TSGs was studied to indicate the methylation status in human cancer. qPCR was performed using the EpiScope® Promoter qPCR Array with SYBR Green-based detection (TaKaRa Bio Inc., Otsu, Japan) and the StepOne™ real-time detection system (Applied Biosystems) according to the manufacturer's protocol. The specificity of targeted PCR product was confirmed by melt-curve analysis, which is essential in an efficient and specific quantitative PCR assay. Alterations in the chromatin associated with damaged DNA (i.e. 8-OHdG-bound DNA elements) before and after the H₂O₂ treatment were also assessed. Samples were run in triplicate and data were normalized to amplifications of 5% input samples. The fold changes in the measures of histone modification and the 8-OHdG level in treated and untreated cells were calculated.

Statistical Analysis

To determine the significant variables that contribute to an increase in the number of methylated TSGs, the χ^2 test was used. Multiple comparisons between the fold changes of AcK16H4, 3MeK4H3, 3MeK27H3, 8-OHdG and that of H3 were done using

Steel's method. The correlation between each fold change (for AcK16H4, 3MeK4H3, 3MeK27H3 and H3) and the 8-OHdG level was evaluated by Spearman's rank correlation test. All p values were calculated employing a two-tailed analysis and $p < 0.05$ was considered statistically significant. All statistical analyses were performed by using JMP version 9.0 software (SAS Institute Inc., Cary, N.C., USA).

Results

Association between ROS-Mediated DNA Damage and TSG Methylation Events in the Liver during CHC Infection

In order to evaluate the ROS-mediated DNA damage, we performed IHC staining of 8-OHdG in 125 out of 128 liver biopsy specimens of CHC tissues. The CHC cases were categorized into 3 subgroups as described in Materials and Methods. Among the 125 specimens, 34 were classified as having a strong staining (2+) of 8-OHdG, 30 as moderate (1+) and 61 as weak ($\pm$). Similarly, 10 specimens showed strong deposits (2+) of iron, 40 moderate (1+) and 75 weak ($\pm$). We also assessed TSG promoter methylation in all the 128 CHC biopsy specimens. Of these, 27 CHC specimens showed methylation in 3 or more TSGs, and 101 specimens showed fewer than 3 methylated TSGs.

The association between each clinicopathological factor and the number of methylated TSGs in the liver tissues is summarized in table 1. Although an increase in the number of methylated TSGs (≥ 3) was more prevalent in CHC of older-aged males (≥ 55 years) with advanced F-stage fibrosis (F2–F4) and strong deposits of iron, the 8-OHdG level in the liver tissues was the only factor that significantly correlated with the increased TSG methylation in CHC ($p < 0.0001$).

Alteration of Histone Modification Induced by H₂O₂ Treatment

The strong relationship between the 8-OHdG level and TSG methylation suggested that the formation of 8-OHdG might induce methylation-associated gene silencing through the formation of repressive histone markers. To test this hypothesis, we used ChIP to measure the fold changes of repressive and active histone markers at the CpG-containing TSG promoters with 8-OHdG before and after H₂O₂ treatment. For this purpose, we performed ChIP-qPCR on the promoters of 30 transcripts in the 25 genes using antibodies against AcK16H4 and 3MeK4H3 (the active histone markers), 3MeK27H3 (the repressive histone marker) and 8-OHdG (to detect damaged DNA elements in the HepG2 cells).

Table 1. Association between the number of methylated TSGs and clinicopathological factors in patients with CHC

Variables	Cases, n	Methylated TSGs ¹ , n		p value ²
		<3	≥ 3	
Age				
<55 years	54	46	8	0.1311
≥ 55 years	74	55	19	
Gender				
Female	43	38	5	0.0522
Male	85	63	22	
F-stage				
F0 or F1	46	39	7	0.2135
F2–F4	82	62	20	
Staining of 8-OHdG				
$\pm$	61	58	3	<0.0001
1+	30	22	8	
2+	34	20	14	
Iron deposit				
$\pm$	75	63	12	0.2191
1+	40	31	9	
2+	10	6	4	

¹ The number of methylated TSGs was determined using MethylLight. Eleven TSGs (*HIC-1*, *GSTP1*, *SOCS1*, *RASSF1*, *CDKN2A*, *APC*, *RUNX3*, *PRDM2*, *CASP8*, *CACNA1G* and *PTGS2*) were analyzed. ² p values were calculated using the χ^2 test.

Among the promoters of the 30 transcripts, we successfully amplified the promoter regions of 29 transcripts using the post-ChIP DNA for the measurement of AcK16H4. Similarly, 25, 27 and 26 promoters could be amplified after the ChIP for 3MeK4H3, 3MeK27H3 and 8-OHdG, respectively. All but one promoter (the *LOX* gene) were amplified after the ChIP for H3. Moreover, 23 promoters were amplified by all 4 ChIP-qPCRs, and the fold changes of the ChIP-qPCR values were determined for these loci using H₂O₂-treated and untreated cells (fig. 2a). In the rabbit IgG-negative control ChIP assay, 10 of the 30 gene promoters were slightly amplified; however, the fold changes for the negative control could not be calculated because of its low levels. The median values (25–75%) of fold changes were 0.70 (0.55–0.90) for AcK16H4, 0.80 (0.60–1.10) for 3MeK4H3, 1.60 (1.30–1.80) for 3MeK27H3, 1.45 (1.00–1.93) for 8-OHdG and 1.10 (0.90–1.40) for H3. For 17 promoters, the 8-OHdG fold changes were more than 1.0, suggesting the increase of 8-OHdG at CpG island-containing TSG promoters after H₂O₂ treatment. Simi-

Fig. 2. Alteration of histone modification and its association with 8-OHdG levels after H_2O_2 treatment at the CpG island-containing promoters of TSGs. **a** HepG2 cells were either treated with or without 250 μM of H_2O_2 for 1 h, followed by ChIP for AcK16H4, 3MeK4H3 as an active histone marker, 3MeK27H3 as a repressive histone marker, 8-OHdG, positive control of H3 or negative control of rabbit IgG. The y-axis indicates the fold changes of ChIP-qPCR values over H3 values between the treated and untreated samples. T/U = Ratio of PCR values of treated/untreated cells. * Indicates the splice variants of the corresponding gene. **b** Comparisons of the fold changes indicated by ChIP-qPCR between the IP of each histone modification or 8-OHdG and that of H3. p values were calculated using Steel's method for nonparametric multiple comparison.

larly, the fold changes of the recessive histone marker 3MeK27H3 were greater than 1.0 for 21 TSG promoters. On the other hand, there was a reduction of the active histone markers AcK16H4 and 3MeK4H3 for 17 and 15 promoters, respectively, after treatment with H_2O_2 (fig. 2a). The correlation between the fold changes of AcK16H4, 3MeK4H3, 3MeK27H3, H3 and that of 8-OHdG were also analyzed (fig. 2b). Although it was not statistically significant, the 8-OHdG fold change comparatively increased to that of H3 ($p = 0.1064$ by Steel's method for nonparametric multiple comparison; fig. 2b). The AcK16H4 and 3MeK4H3 fold change values were significantly lower, and the fold change values for 3MeK27H3 were significantly higher than that of H3 ($p = 0.0003, 0.0056$ and 0.0007 for AcK16H4, 3MeK4H3

and 3MeK27H3, respectively, by Steel's multiple comparison test).

We further examined the relationship between the fold changes of 8-OHdG and active and recessive histone markers using H_2O_2 -treated and untreated fetal liver Hc cells. Although the correlation was not prominent for AcK16H4 ($r = -0.2789$, $p = 0.1507$; fig. 3a), there was a moderate inverse correlation between the fold changes of 8-OHdG and those of active histone markers ($r = -0.5273$, $p = 0.0039$ for 3MeK4H3; fig. 3b). Interestingly, a strong correlation was detected between the fold changes of 8-OHdG and those of recessive histone markers of 3MeK27H3 ($r = 0.7605$, $p < 0.0001$; fig. 3c). As expected, no correlation was observed between the fold changes of 8-OHdG and those of H3

Fig. 3. Correlations between the fold changes of ChIP-qPCR values for histone and 8-OHdG. Correlations between the fold changes of AcK16H4 and 8-OHdG (**a**), 3MeK4H3 and 8-OHdG (**b**), 3MeK27H3 and 8-OHdG (**c**), and H3 and 8-OHdG (**d**). Correlations between each fold change and that of 8-OHdG were evaluated by Spearman's rank correlation test.

(fig. 3d). These findings also indicated that the decrease in active chromatin and the increase in repressive chromatin take place on the 8-OHdG-enriched CpG island-containing TSG promoters, after H_2O_2 treatment.

Discussion

A number of reports suggested that the activation of oncogenes and inactivation of TSGs was a characteristic feature of human cancers, including HCC [3, 4]. We previously reported that the inactivation of TSGs through promoter methylation was more prevalent in HCV-related HCCs than in HCV-negative tumors [13]. On the other hand, it was also reported that oxidative stress could

play a central role in the pathogenesis of CHC, and could increase the risk of HCC development [13, 14]. In this study, we examined whether persistent stimulation by ROS and subsequent DNA damage, indicated by the formation of 8-OHdG, could increase the risk for HCC development through the induction of epigenetic instability in hepatocytes. Here, we demonstrated that the 8-OHdG level was the only factor associated with an increased number of methylated TSGs in the liver of CHC, and that TSGs carrying higher levels of 8-OHdG facilitated the modification of the active chromatin to a repressive form after stimulation by ROS.

So far, several reports have suggested that increased ROS production is observed in CHC and the amount of ROS is associated with the onset of HCC [8, 14, 15]. Ac-

tivation of oxidative stress pathways in noncancerous human liver tissue reportedly predicted the recurrence of HCC in patients who underwent a hepatectomy [16]. ROS could also induce genetic alterations such as point mutations because oxidative DNA damage, indicated by 8-OHdG, could induce DNA base mutations such as G>T/C>A transversions [9]. However, although this type of base mutation is commonly found in HCC, whole genome and exome analyses revealed that the mutational spectrum of HCC is heterogeneous [10]. In addition, so far, the frequencies of common mutations in specific TSGs in HCC were not high; the frequencies of the mutations were around 30 and 15% for *p53* and *β-catenin*, respectively [17–21]. On the other hand, a considerable number of cancer-related genes, such as the *CDKN2A*, *RASSF1A*, *GSTP1* and *APC*, showed an alteration in the methylation status in HCC [13, 21]. Regional hypermethylation of the gene promoters leads to transcriptional inactivation of the corresponding TSGs and hypomethylation could cause increased expression of oncogene and transposable DNA elements, both of which could contribute to carcinogenesis.

Previously, we selected the TSGs that showed a high level of methylation in early HCC, and reported that methylation of these TSGs was a unique marker for early-stage HCV-related HCC [11]. In addition, we found that a number of these methylated TSGs in the liver were significantly associated with the onset of HCC in patients with CHC without a prior history of HCC [11]. In this study, we further clarified that the level of 8-OHdG in the liver was the only factor that showed significant association with the methylated TSG number in the same CHC cohort. Previous reports suggested that the treatment of the HCC cell line by H₂O₂ induced an increase in Snail expression and hypermethylation of the *E-Cadherin* promoter. Snail was also shown to recruit HDAC1 and DNMT1 to the *E-Cadherin* gene [22]. Another report showed that oxidative stress induced by H₂O₂ recruits DNA methyltransferase 1 (DNMT1) to damaged chromatin in colon cancer cell lines [23]. It resulted in relocation of the polycomb repressive protein complex from non-GC-rich to GC-rich areas [23]. Thus, it was reasonable to speculate that oxidative damage led to the formation and relocation of a polycomb repressive complex, which may explain cancer-specific aberrant DNA methylation and transcriptional silencing of TSGs. To explore this possibility, we treated HCC-derived cell lines and fetal liver cells with H₂O₂, and determined the effect of ROS on the modulation of histone. As expected, treatment with H₂O₂ induced the formation of 8-OHdG on

the CpG island-containing TSGs. Interestingly, after treatment the measurement of the repressive histone marker, 3MeK27H3, increased, and that of the active histone markers, AcK16H4 and 3MeK4H4, decreased. It was well known that the polycomb complex could induce repressive histone markers such as 3MeK27H3. The repressive histone marker was associated with the induction of DNA methylation in the corresponding region. On the other hand, our results also indicated that the 8-OHdG fold change using H₂O₂-treated and untreated cells was clearly correlated to that of 3MeK27H3, and inversely correlated to that of AcK16H4 and 3MeK4H4. These facts support the idea that ROS induced an alteration of histone modification to the repressive form at the CpG island-containing TSG promoters and induced hypermethylation through the formation of 8-OHdG, which could lead to inactivation of TSGs by epigenetic mechanisms.

In this study, we clearly demonstrated that the ROS-induced DNA damage could cause the induction of repressive histone markers on TSG promoters. Therefore, it is reasonable to believe that the increased TSG methylation seen in HCC could be reflective of the alteration of chromatin status induced by ROS. As the accumulation of methylated TSGs in CHC tissues has been shown to be a strong risk factor for the early onset of HCC, ROS probably plays a critical role in human hepatocarcinogenesis through the induction of epigenetic instability and regional hypermethylation in CHC.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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