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miR-1247 blocks SOX9–mediated regeneration in alcohol- and fibrosis-associated acute kidney injury in mice☆

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ABSTRACT

Excessive alcohol consumption has a significant impact on human health and is a major public health problem worldwide. One of the consequences of long-term excessive alcohol consumption is cellular injury in almost all organs and tissues, with acute kidney injury (AKI) being one of the most common pathological manifestations. In the present study, using a mouse model of alcoholic liver fibrosis-associated AKI induced by a combined treatment with carbon tetrachloride (CCl_4) and ethanol and resembling pathological features of AKI in human alcoholic liver fibrosis, we demonstrate alterations in histone modifications in the kidneys and, importantly, in the promoter region of the over-expressed SRY (sex determining region Y)-box 9 (*Sox9*) gene. The level of SOX9 protein in the kidneys of AKI-mice is reduced and correlates inversely with increased expression of microRNA miR-1247. Mechanistically, the over-expression of miR-1247 is associated with a markedly increase in histone H3 lysine 4 trimethylation in the upstream region of the *Mir1247* gene. The results of the present study demonstrate a functional role of epigenetic mechanisms in AKI and indicate the importance of correcting the epigenetic dysregulation for proper renal tubule maintenance and repair.

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1. Introduction

Excessive alcohol consumption has a significant impact on human health and is a major public health problem worldwide (World Health Organization, 2014). Long-term excessive alcohol consumption can lead to cellular injury in almost all organs and tissues, with alcoholic liver diseases being the most prominent clinical syndromes (Rusyn and Bataller, 2013; Louvet and Mathurin, 2015). Additionally, it is well-established that excessive alcohol consumption has harmful effects on the kidney (Cecchin and De Marchi, 1996; Epstein, 1997), with acute kidney injury (AKI) being one of the leading causes of severe acute renal failure (relative risk = 4.3, 95% confidence interval = 3.1–5.8) (Bagshaw et al., 2005); it also has been associated with IgA glomerulonephritis, acute nephropathy, and kidney graft failure (Schaeffner and Ritz, 2012). Because alcohol affects multiple organs, co-morbidity plays a critical role in alcohol abuse-associated severity and mortality (Altamirano et al., 2012; Maiwall et al., 2016). For instance, Altamirano et al. (2012) demonstrated that AKI is a frequent and early event in patients with alcoholic hepatitis. Additionally, the prevalence of AKI was reported in patients with advanced liver cirrhosis (Russ et al., 2015; Wong, 2015; Francoz et al., 2016), which is a major pathological consequence of alcohol abuse.

The pathogenesis of AKI is associated with a number of cellular and molecular disturbances, including the induction of oxidative

Abbreviations: NBT, 4-Nitro-blue tetrazolium; CpG, 2′-deoxycytidylyl-(3′→5′)-2′-deoxyguanosine; BCIP, 5-bromo-4-chloro-3′-indolylphosphate; H4K16ac, acetylated lysine 16 histone H4; H3K27ac, acetylated lysine 27 of histone H3; H3K56ac, acetylated lysine 56 of histone H3; H3K9ac, acetylated lysine 9 of histone H3; AKI, acute kidney injury; AP, alkaline phosphatase; CCl_4, carbon tetrachloride; ChIP, chromatin immunoprecipitation; DIG, digoxigenin; Dnmt, DNA methyltransferase; EtOH, ethanol; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *Havcr*, hepatitis A virus cellular receptor 1; HRP, horseradish peroxidase; *Kim1*, kidney injury molecule-1; *Lcn2*, lipocalin 2; MeDIP, methylated DNA immunoprecipitation; miRNA, microRNA; *Ngal*, neutrophil gelatinase-associated lipocalin; ANOVA, analysis of variance; PBS, phosphate-buffered saline; SSC, saline-sodium citrate; Sox9, SRY (sex determining region Y)-box 9; H4K20me3, trimethylated lysine 20 of histone H4; H3K27me3, trimethylated lysine 27 of histone H3; H3K4me3, trimethylated lysine 4 of histone H3; H3K9me3, trimethylated lysine 9 of histone H3; ACTB, β-actin.

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stress, mitochondrial dysfunction, inflammation, autophagy, perturbation in cell signaling pathways, and apoptotic cell death (Havasi and Borkan, 2011; Bonventre and Yang, 2011; Linkermann et al., 2014; Livingston and Dong, 2014; Sureshbabu et al., 2015). In addition to these well-established pathophysiological events, evidence acquired in recent years indicates that AKI is characterized by significant epigenetic abnormalities, including aberrant DNA methylation, histone modification, and microRNA (miRNA) expression (Bomsztyk and Denisenko, 2013; Khalid et al., 2014; Reddy and Natarajan, 2015; Aguado-Fraile et al., 2015); however, there is a lack of knowledge regarding (i) the significance of these changes in AKI and (ii) the mechanistic relationship between different individual epigenetic components in the pathogenesis of AKI.

This study was designed to elucidate the role of epigenetic mechanisms in alcoholic liver fibrosis-associated AKI. To achieve this goal, we employed a recently developed fibrosis- and alcohol-associated mouse model of AKI that resembles distinct pathological features of AKI in human alcoholic liver fibrosis (Furuya et al., 2016). We demonstrate that alcoholic liver fibrosis-associated AKI in mice was characterized by profoundly altered histone modifications in the kidneys, in particular, by markedly increased levels of global acetylation at lysine residues 27 and 56 of histone H3 (H3K27ac and H3K27ac) and lysine 16 of histone H4 (H4K16ac), and trimethylation at lysine residues 4, 9, and 27 of histone H3 (H3K4me3, H3K9me3, and H3K27me3) and lysine 20 of histone H4 (H4K20me3). Among these histone modifications, the greatest changes were found in the extent of H3K4me3, a mark indicative of transcriptional activation. Importantly, the promoter region of the over-expressed SRY (sex determining region Y)-box 9 (*Sox9*) gene, an acute epithelial stress response and key renal tubule regeneration gene in AKI (Kumar et al., 2015; Kang et al., 2016), was greatly enriched in H3K4me3 and depleted in H3K27me3. Surprisingly, the level of SOX9 protein in the kidneys, especially renal tubules, in AKI-mice was dramatically reduced and correlated with an increased expression of several miRNAs that target directly *Sox9* mRNA, most prominently miR-1247. Mechanistically, the over-expression of miR-1247 was associated with a significant enrichment of H3K4me3 in the upstream region of the *Mir1247* gene. The results of the present study demonstrate a critical functional role of epigenetic mechanisms in the cellular and molecular processes involved in AKI and indicate the importance of correcting of epigenetic dysregulation for the proper renal tubule maintenance and repair.

2. Materials and methods

2.1. Animals, experimental design, and treatments

Male C57BL/6J mice (12 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME). The study design, mouse treatment, tissue sample collection, and kidney histopathology are detailed in Furuya et al. (2016). Briefly, mice were allocated randomly to one control and three experimental groups. Mice in "Control" and "EtOH" groups received intraperitoneal (*i.p.*) injections of olive oil vehicle, 2 times per week, for 6 weeks. Mice in "CCl_4" and "CCl_4 + EtOH" groups received *i.p.* injections of 200 μL CCl_4/kg body weight (bw), 2 times per week, for 6 weeks. After 6 weeks of treatment, all mice underwent surgical intra-gastric intubation (Kono et al., 2000). After 1 week of recovery, mice in "Control" group were maintained on normal animal chow for 3 weeks; mice in "CCl_4" group received *i.p.* injections of 100 μL CCl_4/kg bw, 2 times per week, for 3 weeks; animals in "EtOH" group were administered EtOH intragastrically for 3 weeks; and mice in "CCl_4 + EtOH" were administered EtOH intragastrically and *i.p.* injections 100 μL CCl_4/kg bw, 2 times per week, for 3 weeks. Upon the euthanasia, the kidneys were excisedm and sections of the kidney fixed in 10% neutral buffered formalin. The remaining kidney tissues were snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analyses. Frozen and formalin-fixed/paraffin-embedded kidney tissue samples from this study were used for the experiments detailed below.

2.2. Western blotting

The levels of H3K4me3, H3K9me3, H3K27me3, H4K20me3, H3K9ac, H3K27ac, H3K56ac, H4K16ac, and SOX9 in the kidneys were determined by Western blot analysis. Primary antibodies against H3K27ac, H3K9me3, H3K27me3, and H4K20me3 were obtained from Millipore Corporation (Billerica, MA, USA); against H3K9ac, H3K56ac, H4K16ac, and SOX9 from Abcam (Cambridge, MA, USA); and against H3K4me3 from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies were diluted 1:1000 or 1:2000 according to the manufacturers' recommendations. IRDye 800CW anti-rabbit or IRDye 680RD anti-mouse secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used for visualization. Fluorescence was measured directly with an Odyssey CLx Infrared Imager (LI-COR Biosciences). Signal intensities were analyzed using ImageQuant 4.0 software (Molecular Dynamics, Sunnyvale, CA, USA). Equal protein loading was confirmed by immunostaining against the total H3 (Millipore Corporation) and total H4 (Abcam) or β-actin (ACTB; Abcam).

2.3. DNA isolation and analysis of global genomic DNA methylation

Genomic DNA was extracted from kidney tissues using DNeasy Blood and Tissue Mini kits following the manufacturer's instructions (Qiagen, Valencia, CA, USA). The methylation status of genomic DNA was evaluated by a methylation sensitive HpaII/MspI-based cytosine extension assay as detailed in Pogribny et al. (2004). The methylation status of short interspersed nuclear elements (SINE B1 and SINE B2) was determined by quantitative PCR (qPCR) using input and immunoprecipitated DNA according instructions for MethylMiner Methylated DNA Enrichment kits (Invitrogen, Carlsbad, CA, USA).

2.4. Total RNA isolation and qRT-PCR

RNA was isolated from kidney tissues using miRNeasy Mini kits (Qiagen) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using random primers and High Capacity cDNA Reverse Transcription kits (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol and gene expression was determined by quantitative reverse-transcription PCR (qRT-PCR) using the TaqMan gene expression assays (Life Technologies). The following assays were used for qRT-PCR: DNA methyltransferase 1 (*Dnmt1*; Mm01151063_m1), *Dnmt3a* (Mm00432881_m1), *Dnmt3b* (Mm01240113_m1), and *Sox9* (Mm00448840_m1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Mm99999915_g1) was used as an endogenous control. The relative amount of each mRNA transcript was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.5. Methylated DNA immunoprecipitation assay

Methylated DNA immunoprecipitation (MeDIP) was performed with MethylMiner Methylated DNA Enrichment kits (Invitrogen) according to the manufacturer's instructions. The methylation status of the CpG islands located within the promoter/first exon region of the *Sox9*, *Mir145*, and *Mir1247* genes was determined by quantitative PCR (qPCR) of DNA from immunoprecipitated and unbound DNA. The primer sets for the MeDIP analysis of the *Sox9*,