Genetic Factors of Alcoholic Steatohepatitis

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Abstract:

Chronic, excessive alcohol consumption is the basis for alcoholic liver disease (ALD), the leading cause of illness and death from liver complications in the United States. Specifically, alcoholic steatohepatitis (ASH) is a type of liver disease characterized by the accumulation of fat in liver (steatosis) in conjunction with liver inflammation (hepatitis). Of the patients that develop hepatitis, only a portion progress to further liver damage in the forms of fibrosis and cirrhosis. The mechanism of progression from early fat accumulation to more severe fibrosis and cirrhosis, however, is not fully understood.

Previous studies have provided evidence to suggest that the development and progression of ASH is a polygenic disease, involving multiple genetic and environmental risk factors. A recent study identified a single nucleotide polymorphism (SNP) in DDX5, an ATP-dependent RNA helicase and transcriptional modifier. This missense single nucleotide polymorphism increased fibrogenic gene transcription in hepatic stellate cells, providing evidence that SNPs are capable of modulating cell specific mechanisms related to liver injury. In our study, we aimed to identify SNPs in target gene NLR family, CARD domain containing 4 (Nlrc4). Using Primer3Plus, we designed sets of primers to amplify a 1kbp fragment in the promoter region upstream of the first exon in both Noxo1 and Nlrc4. Utilizing an Illumina Beadstation 500GX Genetic Analysis System to sequence the amplification products, we found a single nucleotide deletion, specific to A/J mice, 330bp upstream in the Nlrc4 promoter region.

Further study of the mutation was conducted through in vitro procedures. The Nlrc4 promoter region was inserted in to HepG2 and RAW 264.7 cell lines, and expression levels were evaluated using a luciferase kit. We found that the mutation in the promoter region down regulated gene expression in hepatocytes (HepG2) and up regulated expression in macrophages (RAW 264.7). With this data, we believe that the mutation results in reduced cell death of hepatocytes, while also causing a reduction in release of inflammatory markers from macrophages. These two factors combined lead to an overall decrease in activation of hepatic stellate cells, and ultimately, development of fibrosis. As we continue our investigation, we aim to this mutation and gene with the human analog. It is known that there is a single nucleotide polymorphism in the promoter region of the Nlrc4 gene, but the effects of the mutation are unknown. We also are examining the role of Cdx1 in liver regeneration. We hope to better understand the roles of Nlrc4, Cdx1, and the overall molecular pathway that contributes to the progression of liver disease.

Background:

It is known that the liver is a primary target of alcohol related injury. Though complete details of alcoholic liver steatohepatitis is not understood, it has been seen that increased production of reactive oxygen species (ROS) is related to the development of alcoholic liver disease (1; 2; 3). An important member in the process of ROS damage is NADPH oxidase. NOX family of NADPH oxidases are proteins involved in the transfer of electrons across biological membranes.
Typically, oxygen acts as an electron acceptor while the product of the electron transfer reaction is superoxide. The generation of ROS not only is involved in cellular damage, but is also a factor in many reversible regulatory processes (4). Of the several members in the NOX family, there are two NOX organizer subunits that are known: p47phox, and Noxo1. Noxo1 expression has been found in the testis, colon, liver, kidney, and pancreas (5). Among other proteins, these NOX subunits are required for activity of NADPH oxidase. Loss of the organizer proteins interfere with NADPH oxidase activity. For example, mice deficient for p47phox are resistant to chronic ethanol related injury (6) while mice with an inactive Noxo1 mutation leads to arrest of inner ear formation resulting in severe imbalance (7). There is little data on the promoter of Noxo1, and there are no published data for polymorphisms found in p47phox, Noxo1, or NoxA1 in humans. In our study, we aimed to locate SNPs in the promoter of Noxo1, and identify the downstream effects of any such mutations.

A major factor in the pathogenesis of liver disease is activation of the immune system. Currently hypothesis of ethanol related liver injury suggests that ethanol causes leakage of bacterial products from the gut. Long term exposure to alcohol leads to an increase of gram negative bacteria, in turn increasing circulation of lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria, among alcoholics (8). Microbial structures,
referred to as pathogen associated molecular patterns (PAMP), are identified in the body by membrane bound Toll-like receptors (TLR) and cytoplasmic nucleotide oligomerization domain like receptors (NLR) (9). In presence of PAMPs, NLR interacts with members of the inflammasome complex. Downstream effects of this interaction include promotion of proteolytic maturation as well as mediation of apoptotic cell death. Currently there are no data base entries for SNPs in Nlrc4 in humans. Like Noxo1, we aimed to identify SNPs in the promoter region of Nlrc4 and determine downstream effects of these mutations.

Methods:

To identify target genes in alcohol related liver injury, we first needed to develop and confirm pairs of congenic mouse strains (CSS) developed by Dr. Joseph Nadeau. CSS strains have one chromosome from A/J inbred strain that has been substituted for the corresponding chromosome in the B6 genetic background. These strains allow for a comparison of injury differences between A/J and B6 specific to chromosome 17. After confirmation of congenic strains through genotyping PCR, we treated the mice with a 4-week ethanol containing or pair-fed diet. Food intakes were measured daily, and diet was switched out daily in both groups.

In order to quantify liver injury after treatment with diet, Oil Red O staining of liver sections were prepared to determine degree of steatosis in each mouse. Liver triglycerides were measured biochemically with Triglyceride (GPO) reagent set from Pointe Scientific (Lincoln Park, MI). ALT and AST plasma levels were measured enzymatically in mice using a commercially available kit (Diagnostic Chemical, Ltd, Oxford, CT). Triglyceride, ALT, and AST levels are all indicators that help quantify the extent of liver injury. After identifying liver injury, we used a deep short read sequencing process to identify potential genes involved with alcoholic liver disease. RNA was isolated from each mouse after the 4 week diet, and expression levels of mRNA was determined using RT-PCR, normalized against 18s rRNA. This process allowed us to determine target genes Nlrc4 and Noxo1.

After determining Nlrc4 and Noxo1 as target genes, it was necessary to isolate and amplify a 1kb promoter region of each gene. The promoter regions were then sequenced and analyzed to determine SNPs and surrounding consensus binding sites. Primers were designed using Primer3Plus, and the region was amplified through PCR. An illumina Beadstation 500GX Genetic Analysis System was utilized to sequence the 1kb fragment. Sequencing data were analyzed and aligned with Sequence Scanner v1.0 and BioEdit v7.0.0, while consensus binding sites were determined using TFSEARCH: Searching for Transcription Factor Binding Sites (ver1.3). After sequencing and identifying the mutation, we ligated the 1kb promoter regions into plasmids containing the reporter gene pGL3 luciferase. These plasmids were then transfected into competent cells, and the cells were let to grow. Expression levels of the luciferase reporter gene were measured and compared between the A/J and B6 promoter insertions. Our next steps include obtaining sequences for the human promoter region of Nlrc4, and determining the single nucleotide polymorphism in the region. This region would then be inserted into competent cells, and gene expression levels would be evaluated as before.
Additionally, we would like to examine the regenerative properties of Cdx1. This involves inducing liver damage in mouse models, and determining Cdx1 expression levels at various time points. Liver damage can be induced via ethanol diet, or a cycle of CCl4 injections. Cdx1 expression levels will be evaluated using qPCR.

Results:

From administration of chronic Lieber DeCarli ethanol diet, or treatment with CCl4, it was seen that one region of A/J chromosome 17 (17C-1 strain) promotes fibrosis and alcohol-induced steatosis. In the ethanol study, all mice had increased steatosis and elevated ALT/AST levels on Lieber DeCarli ethanol diet when compared with pair-fed counterparts. Uniquely, 17C-1 strain had greater steatosis and increased ALT levels in both ethanol and pair-fed diets when compared to B6 mice.

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
<th>CSS-17</th>
<th>17C-1</th>
<th>17C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF</td>
<td>11.7±0.1a</td>
<td>12.5±0.4b</td>
<td>13.1±0.1b</td>
<td>13.0±0.3b</td>
</tr>
<tr>
<td>+E</td>
<td>12.5±0.4b</td>
<td>13.1±0.1b</td>
<td>12.0±0.1b</td>
<td>13.2±0.3b</td>
</tr>
<tr>
<td>Liver TG (mg/g liver)</td>
<td>35.2±2.7a</td>
<td>77.9±9.4b</td>
<td>32.1±2.6b</td>
<td>57.6±4.4c</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>18.5±1.5a</td>
<td>32.3±3.5b</td>
<td>16.3±0.9b</td>
<td>22.2±1.9c</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>62.0±7.6a</td>
<td>75.2±5.4a</td>
<td>55.4±6.3c</td>
<td>64.4±7.3a</td>
</tr>
</tbody>
</table>

Table 1. Food Intake, Hepatic triglycerides and ALT/AST levels in B6,CSS-17 and congenic mice.

Mice were chronically fed ethanol-containing diet (+E) or pair-fed (PF) for 4 weeks. Food intakes were measured daily and the measurements are the averages over the course of the feeding trial. Liver triglycerides were measured biochemically with Triglyceride (GPO) reagent set from Pointe Scientific (Lincoln Park, MI). ALT and AST plasma levels were measured enzymatically in mice using a commercially available kit (Diagnostic Chemical, Ltd, Oxford, CT). Values are the mean ± SEM for n=4-6 mice. The means in a row with superscripts without a common letter differ *P*<0.05 as determined by ANOVA analysis.

In the CCl4 treatment model, significant fibrosis was seen in 17C1 mice, while B6 showed moderate development of fibrosis. A/J and 17C6 mice had little fibrosis, and seemed to be protected. From these results, we were able to identify one region of A/J chromosome that promotes fibrosis (17C1), while the distal region protects from CCl4-induced fibrosis.
To identify candidate genes in the regulation of ASH, we conducted a deep short-read sequence of chromosome 17 from B6 and A/J mouse strains. Chromosome 17 was sequenced to 22 fold coverage (22x) to identify nucleotide polymorphisms, short indels, and structural variants with a high degree of sensitivity and accuracy. Among several genes identified with the above mutations, two genes Nlrc4 and Noxo1 were found to be strong candidates in liver damage mediation. These two genes were refined by measuring gene expression levels in liver samples of mice treated with the Lieber DeCarli ethanol diet or pair-fed diet for 4 weeks. After analyzing expression level differences in B6, A/J, and congenic strains, it was seen that Noxo1 expression increased after administration of Lieber-DeCarli ethanol diet or CCl\textsubscript{4} treatment in congenic strains 17C-1 and CSS-17. It was also found that Nlrc4 expression was reduced in mice on the ethanol diet, however, in strains that were protected from alcohol related liver damage (17C-6 and CSS-17) Nlrc4 mRNA expression was higher when compared to non-protected B6 and 17C-1 strains.

Analysis of sequencing data of a 1kb promoter region in Nlrc4 showed a single nucleotide deletion in Nlrc4. The deletion was found 331bp
upstream of the first exon in the promoter region of Nlrc4 specific to A/J mice.

Deletion specific to A/J @ 331bp upstream

A/J promoter for Nlrc4

\[
\text{TATTATTATTTTTTTTTTTATGTATAAGTAGTACACTGTTGCTTCC}
\]

B6 promoter for Nlrc4

Extra bp present 331bp upstream in B6

\[
\text{TATTATTATTTTTTTTTTTATGTATAAGTAGTACACTGTTGCTTCC}
\]

Cdx1 - - - - - - -

The deletion mutation occurs specifically at the Cdx1 binding site in the promoter region. Further analysis of this deletion (through in vitro studies) showed that the mutation had significant effects on down stream gene expression in both hepatocytes and macrophages.

\[\text{Cdx-1 is overexpressed}\]

Statistics measured with ANOVA

In hepatocytes, we see that the deletion at the Cdx1 binding site results in down regulated gene expression.

\[\text{Cdx-1 is overexpressed}\]

Statistics measured with ANOVA

The opposite was seen in macrophages, were the mutation actually resulted in up regulated expression.
Discussion:

Elevated ALT levels and greater steatosis found in 17C-1 congenic strain suggests that the 27Mb region of A/J in 17C-1 strain has an increased susceptibility to alcohol induced injury. Further data from treatment with CCl₄ showed that significant fibrosis developed in the 17C-1 strain. It is reasonable to conclude that certain genes in the 17C-1 region are involved in the regulation of liver damage. Upon analysis of the 17C-1 congenic strain, Noxo1 was identified as a strong candidate gene for ASH and the modulation of apoptosis. Amplification and sequencing of the Noxo1 promoter region showed a SNP that may affect several surrounding consensus binding sites. Further analysis of Noxo1, through ligation into plasmid and transfection into competent cells, will allow us to determine the role of Noxo1 in the alcoholic liver disease pathway.

CCl₄ injection treatment also showed that the distal region 17C-6 strain was protected from liver fibrosis. Additionally, in strains protected from alcohol related injury (B6, 17C-1), we saw an elevation in Nlrc4 mRNA expression. It is likely that Nlrc4 has a significant role in resistance to alcohol induced liver damage. Deep sequencing analysis of the 17C-6 strains revealed that Nlrc4 is a strong candidate gene in the development of ASH. Further amplification and sequencing of the Nlrc4 promoter region showed a single nucleotide deletion specific to A/J mice that may significantly affect several consensus binding sites within the region. This data suggests that the deletion in the promoter region may have significant implications in Nlrc4 function between B6 and A/J mice. Further analysis of Nlrc4 expression under varying conditions will allow for a better understanding of the role of Nlrc4 in alcoholic liver disease progression.

Specific analysis of the deletion mutation found in Nlrc4 showed that there were significant down stream effects on gene expression. These effects also seemed to vary greatly between types of liver cells. In hepatocytes, the mutation cause gene expression to be significantly lowered, while in macrophages, gene expression was significantly elevated. This data leads us to believe that the mutation at the Cdx1 binding site in the promoter region of Nlrc4 may be responsible for the overall general resistance to alcoholic liver damage seen in A/J, and related strains of mice.

In mice with the deletion mutation, Nlrc4 will be down regulated in hepatocytes. Because Nlrc4 is involved in the inflammatory response, and cell apoptosis, a down regulation of Nlrc4 leads us to believe that there will be a significant reduction in hepatocyte apoptosis. At the same time, the increased Nlrc4 expression in Kupffer cells will lead to greater apoptosis of the macrophage cells. This increased cell death of the macrophage cells, however, will ultimately lead to a reduction in release of inflammatory markers. A reduction in both these factors, hepatocyte apoptosis, and inflammatory marker release, will overall provide fewer markers for hepatic stellate cells to react to. A decrease in HSC activation will ultimately result in less fibrosis, and lead to the overall resistance to alcoholic liver damage seen in mice with the deletion mutation.
We see here an overall pathway of the mutation and its effects. The mutation in Nlrc4 promoter leads to decreased apoptosis of hepatocytes and increased apoptosis of Kupffer cells. With less functioning Kupffer cells, inflammatory markers are reduced. Lowered apoptotic bodies with lowered inflammatory markers reduces activation of hepatic stellate cells, ultimately leading to reduced fibrosis and liver damage.

**Conclusion:**

In order to better understand the genetic factors of alcoholic liver disease, we used several congeneric mouse strains of chromosome 17 in our study. These strains were treated in various ways to induced liver damage, such as with Lieber DeCarli ethanol diet, or with injection of CCl₄. Analysis of liver injury from these treatments allowed us to identify several regions on chromosome 17 that may be involved with liver damage regulation. We determined two candidate genes Nlrc4 and Noxo1 that seem to have a major involvement in the progression of alcohol related injury. Sequencing of the amplification products revealed a single nucleotide deletion, specific to A/J mice, 330bp upstream in the Nlrc4 promoter region. Likewise, a missense SNP was found 819bp upstream in the Noxo1 promoter region. These mutations may significantly affect consensus binding sites within the promoter regions, suggesting that differences in Nlrc4 and Noxo1 between B6 and A/J mice may contribute to differences in sensitivity to alcohol induced injury. As we
continue our investigation, we aim to identify the downstream effects of these polymorphisms.

We were successful in constructing two plasmids, each containing the promoter for Nlrc4 or Noxo1, and the reporter gene pGL3 luciferase. Transfection of these plasmids into competent cells allowed us to evaluate expression levels in specific environments. We saw that the mutation causes a down regulation in gene expression in hepatocytes and an up regulation of gene expression in Kupffer cells. These significant changes together result in lowered markers for hepatic stellate cells to react to. A reduction in HSC activation ultimately leads to reduced fibrosis, and resistance to alcoholic liver damage. By continuing research into the molecular pathway of alcoholic liver disease, and the specific role of Nlrc4 in the liver, we hope to better understand the nature of the disease. It may be possible to identify targets in the molecular pathway for therapeutic treatment or intervention. We will continue to evaluate the promoter region mutation in the human Nlrc4 gene, and hope to determine whether or not the mutation has significant downstream effects. Additionally, we will further investigate Cdx1, and the role that this protein may play in liver regeneration. It is possible that Cdx1 may aid in liver health not through initial resistance, but rather through increased regeneration. We believe this research may one day lead to a prevention or improvement to the treatment of chronic liver disease in humans.
References: