Deletion of Nardilysin Prevents the Development of Steatohepatitis and Liver Fibrotic Changes (Dissertation)
Deletion of Nardilysin Prevents the Development of Steatohepatitis and Liver Fibrotic Changes

Shoko Ishizu-Higashi¹, Hiroshi Seno¹*, Eiichiro Nishi²*, Yoshihide Matsumoto¹, Kozo Ikuta¹, Motoyuki Tsuda¹, Yoshito Kimura¹, Yutaka Takada¹, Yuto Kimura¹, Yuki Nakanishi¹, Keitaro Kanda¹, Hideyuki Komekado¹, Tsutomu Chiba¹

¹Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan, ²Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan

Abstract

Nonalcoholic steatohepatitis (NASH) is an inflammatory form of nonalcoholic fatty liver disease that progresses to liver cirrhosis. It is still unknown how only limited patients with fatty liver develop NASH. Tumor necrosis factor (TNF)-α is one of the key molecules in initiating the vicious circle of inflammations. Nardilysin (N-arginine dibasic convertase; Nrd1), a zinc metalloendopeptidase of the M16 family, enhances ectodomain shedding of TNF-α, resulting in the activation of inflammatory responses. In this study, we aimed to examine the role of Nrd1 in the development of NASH. Nrd1−/− and Nrd1+/− mice were fed a control choline-supplemented amino acid-defined (CSAA) diet or a choline-deficient amino acid-defined (CDAA) diet. Fatty deposits were accumulated in the livers of both Nrd1−/− and Nrd1+/− mice by the administration of the CSAA or CDAA diets, although the amount of liver triglyceride in Nrd1−/− mice was lower than that in Nrd1+/− mice. Serum alanine aminotransferase levels were increased in Nrd1−/− mice but not in Nrd1+/− mice fed the CSAA diet. mRNA expression of inflammatory cytokines were decreased in Nrd1−/− mice than in Nrd1+/− mice fed the CDAA diet. While TNF-α protein was detected in both Nrd1−/− and Nrd1+/− mouse livers fed the CDAA diet, secretion of TNF-α in Nrd1−/− mice was significantly less than that in Nrd1+/− mice, indicating the decreased TNF-α shedding in Nrd1−/− mouse liver. Notably, fibrotic changes of the liver, accompanied by the increase of fibrogenic markers, were not observed in Nrd1−/− mice but not in Nrd1+/− mice fed the CDAA diet. Similar to the CDAA diet, fibrotic changes were not observed in Nrd1+/− mice fed a high-fat diet. Thus, deletion of nardilysin prevents the development of diet-induced steatohepatitis and liver fibrogenesis. Nardilysin could be an attractive target for anti-inflammatory therapy against NASH.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a condition in which excess fat accumulates in the hepatocytes of patients without a history of alcohol abuse [1]. NAFLD is a hepatic manifestation of metabolic syndromes, such as obesity, type-II diabetes mellitus, and hyperlipidemia. Its prevalence is increasing particularly in the developed countries [1,2]. Nonalcoholic steatohepatitis (NASH) is a severe form of NAFLD, in which liver inflammation is observed and which progresses to liver fibrosis. A part of NAFLD patients develops NASH that leads to liver fibrosis. However, the exact causes and mechanisms of the development of NASH remain unknown.

Recent investigations have suggested a “multi-hit process” model for the development of NASH [3]. Liver inflammation including NASH can be initiated or enhanced by multiple cytokines secreted mainly by Kupffer cells or macrophages [4]. During liver fibrogenesis, myofibroblasts, that are not present in normal liver, also contribute to liver fibrogenesis through the remodeling of extracellular matrix [5]. In pro-inflammatory cascades, there are several key factors that play a crucial role in initiating or halting inflammation. Tumor necrosis factor (TNF)-α is one of such key molecules, and anti-TNF-α therapies are used widely to treat human inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel diseases [6,7]. To activate TNF-α, a membrane-bound pro-TNF-α must be appropriately and sufficiently cleaved by the prototypical sheddase, TNF-α-converting enzyme (TACE) [8]. Previously, we showed that nardilysin (N-arginine dibasic convertase; Nrd1), a zinc metalloendopeptidase of the M16 family that ubiquitously localizes in various organs, enhances the shedding of TNF-α through TACE activation [9–13]. Nardilysin binds to TACE and directly enhances its catalytic activity [10,11]. It also promotes the ectodomain shedding of...
TNF-α, resulting in activation of the TNF-α/nuclear factor-κB pro-inflammatory signaling cascade [12].

In this study, we aimed to elucidate the mechanisms that distinguish NASH from simple liver steatosis. We examined the role of nardilysin, that is known to enhance TNF-α shedding, in the development of steatohepatitis using Nrd1+/+ and Nrd1−/− mice fed a choline-deficient and amino acid-defined (CDAA) diet and a high-fat diet (HFD), that are used widely to reproduce the natural course of NASH and liver fibrosis in mice as well as in humans.

Materials and Methods

Ethics statement

All animal experiments were undertaken in accordance with institutional guidelines. The Review Board of Kyoto University granted ethical approval for this study.

Animal models

Nardilysin-deficient (Nrd1−/−) mice (CDB0466K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) were previously described [13]. Male Nrd1+/+ and Nrd1−/− mice with the BL6/CBA background were bred and housed in a temperature- and light-controlled facility with unlimited access to food and water. To induce steatohepatitis and liver fibrotic changes, 10–12-week old male mice were fed a control choline-supplemented amino acid-defined (CSAA) diet or a choline-deficient amino acid-defined (CDAA) diet (Research Diets, New Brunswick, NJ, USA) for 4, 12, or 20 weeks according to the previous reports [14,15]. As another diet-induced model of steatohepatitis and liver fibrosis, mice were fed a HFD (Oriental Bio Service, Kyoto, Japan) for 20 weeks on the basis of previous studies [16,17]. Triglyceride levels in the livers were determined with Triglyceride Quantification kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. Serum levels of alanine aminotransferase (ALT) were
measured using a Transaminase CII-Test Wako kit (Wako Pure Chemical Industries, Osaka, Japan).

**Histological analyses and immunostainings**

The liver was resected at various time points, fixed with 4% buffered paraformaldehyde solution, embedded in paraffin, and sectioned into 5-μm thickness. Oil red O (Wako Pure Chemical Industries) staining was performed to confirm fatty deposition. Sirius red (saturated picric acid containing 0.1% Direct Red 80 and 0.1% Fast Green FCF; Sigma-Aldrich, St. Louis, MO, USA) staining was done to visualize collagen deposition. Stained fibrotic areas were measured as percentage area in a representative ×100 high-power field in each mouse using Image J software. For the immunostainings the sections were incubated overnight with the primary antibodies at 4°C, after which the secondary antibodies were added. Kupffer cells or macrophages were stained with rat anti-F4/80 monoclonal antibody (Abcam). Negative controls were prepared with isotype IgG.

**Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA, USA). Single-strand complementary DNA (cDNA) was synthesized using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Basel, Switzerland), qRT-PCR was performed using SYBR Green I Master (Roche Applied Science) and Light Cycler 480 (Roche Applied Science). Values are expressed as arbitrary units relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sets used were: TNF-α-Forward, CCCTCAGACTGAGTACACGAG; TNF-α-Reverse, GCTAGACGTTGCTGACAG; interleukin (IL) 6-Forward, TACTTCGTTCCATACCCAAATTG; IL6-Reverse, ATCTTTTGGGGTGCTGCAACT; IL1-β-Forward, ATCTTTTGGGGTGCTGCAACT; IL1-β-Reverse, ATCTTTTGGGGTGCTGCAACT; CCR2-Forward, ATCCGACGCTACTATACAG; CCR2-Reverse, ATCGACGATCAGTACAGTACAG; collagen I-For-
Measurement of cytokine levels by enzyme-linked immunosorbent assay (ELISA)

To determine the production and secretion of TNF-α protein in CDAA-treated mouse liver, a modified protocol that described in previous reports was used [18,19]. In brief, a liver fragment was divided into two specimens (100 µg each). One specimen was subjected directly to protein extraction, and the amount of protein extracted was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The other was cultured in a 24-well flat-bottomed culture plate in serum-free Dulbecco’s modified Eagle’s medium (D-MEM; Life Technologies) supplemented with penicillin and streptomycin (Life Technologies). After 12 hours, the supernatant was collected and the protein level measured. The amounts of TNF-α, IL6, and IL1-β proteins were measured using a Mouse ELISA Ready-SET-Go! kit (eBioscience, San Diego, CA, USA) according to the manufacturer’s protocol.

Mouse peritoneal macrophage experiments

Mouse peritoneal macrophages were isolated from 8-week-old female C57BL/6J mice. Peritoneal cells were harvested by peritoneal lavage with 10 ml PBS. Cells were re-suspended and
cultured in D-MEM supplemented with 10% FCS, 100 mg/ml of penicillin, 100 mg/ml of streptomycin, and 1.25 μg/ml of amphotericin B. 1.0×10⁶ peritoneal cells were seeded into a 48-well dish, and incubated for 2 hours. Then, cells were washed in PBS, and re-cultured in the serum-free medium. To inhibit TNF-α activity, either control serum or 0.4 mg/ml of anti-TNF-α neutralizing polyclonal antibodies (R&D systems) was administered into the culture medium. After 30 minutes later, 1 μg/ml of lipopolysaccharide (LPS) were added. Medium and cells were collected 2 hours after the stimulation, and subjected to the analyses according to the methods described above.

**Statistical analyses**

Results are the mean ± standard deviation unless stated otherwise. Differences between treatments, groups, and strains were analyzed using the two-tailed Student’s t-test.

**Results**

*Nrd1−/−* mice did not develop steatohepatitis with CDAA diet

The CDAA diet is deficient in choline only, but contains methionine, allowing observation of the sequential development of steatohepatitis and liver fibrotic changes in a longer experimental...
period in mice [4,14]. The control CSAA diet also causes mild steatosis, but does not result in steatohepatitis and liver fibrotic changes in mice [4,14]. To study the role of nardilysin during the development of steatohepatitis followed by liver fibrosis, mice were fed the CSAA or CDAA diets. Histology and oil red O staining showed that fat accumulation in the livers of both Nrd1+/+ and Nrd1/−/− mice occurred during administration of the CSAA or CDAA diets and increased in a time-dependent manner, although fat accumulation in Nrd1+/+ mice was more prominent than that in Nrd1/−/− mice (Figure 1A). Size of fat deposition was greater in Nrd1+/+ mice than in Nrd1/−/− mice in both diet groups at each time point (Figure 1A), and triglyceride levels in the liver were significantly higher in Nrd1+/+ mice (Figure 1B). There was no significant difference in the liver/body weight ratio between Nrd1+/+ and Nrd1/−/− mice (Figure 1A). However, serum ALT levels were significantly increased in Nrd1+/+ mice upon administration of the CDAA diet, whereas they were not increased in Nrd1/−/− mice fed the CDAA diet (Figure 2A). Serum ALT level was elevated in neither Nrd1+/+ nor Nrd1/−/− mice fed the CSAA diet (Figure 2A). Consistent with these findings, qRT-PCR showed that mRNA expression of inflammatory cytokines, such as IL6 and IL1-β, was significantly increased only in Nrd1+/+ mice fed the CDAA diet when fat accumulation and elevation of ALT were prominent, whereas they were not increased in Nrd1/−/− mice fed the CDAA diet, and in both Nrd1+/+ and Nrd1/−/− mice fed the CSAA diet (Figure 2B). These data indicated that nardilysin played an important role in the development of steatohepatitis and accompanied the production of inflammatory cytokines in mice fed the CDAA diet.

**Nrd1 was required for sufficient secretion of TNF-α**

TNF-α is one of the key molecules that are involved in the development of NASH [4–7,20]. Because secretion of activated TNF-α is the initial step in nardilysin-mediated production of inflammatory cytokines [12], we hypothesized that sufficient secretion of TNF-α by nardilysin is required for the development of steatohepatitis. Thus, we aimed to ascertain whether TNF-α was produced and secreted sufficiently in the livers of Nrd1+/+ and Nrd1/−/− mice fed the CDAA diet. qRT-PCR showed that the mRNA of TNF-α was increased in both Nrd1+/+ and Nrd1/−/− mice fed the CDAA diet, and that in contrast to the results looking at IL6 and IL1-β mRNA levels, there was no significant difference between Nrd1+/+ and Nrd1/−/− mice (Figure 2B). Immunohistochemistry showed that TNF-α protein was detected in F4/80-positive Kupffer cells or macrophages in both Nrd1+/+ and Nrd1/−/− mice fed the CDAA diet for 20 weeks (Figure 3A, right, arrowheads). Conversely, TNF-α protein was barely detected in F4/80-positive Kupffer cells or macrophages in both Nrd1+/+ and Nrd1/−/− mice fed the CSAA diet for 20 weeks (Figure 3A, left). The number of F4/80-positive cells/100 high power field (HPF) in the liver was slightly increased only in Nrd1+/+ mice fed the CDAA diet but not in Nrd1/−/− mice fed the CSAA diet and those

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**Figure 5. Liver fibrotic area was not observed in Nrd1/−/− mice fed the CDAA diet.** A. Liver fibrosis was determined by Sirius red staining (red) in Nrd1+/+ and Nrd1/−/− mice at 4 (upper), 12 (middle), and 20 (lower) weeks in the livers of Nrd1+/+ and Nrd1/−/− mice fed the CSAA or CDAA diet. Fibrotic changes were not observed in Nrd1+/+ or Nrd1/−/− mice fed the CSAA diet (left). Fibrotic changes were prominent in Nrd1+/+ mice, but not in Nrd1/−/− mice fed the CDAA diet (right). Bars indicate 100 μm. B. Quantification of fibrotic areas. Fibrotic areas was observed and increased in a time-dependent manner only in the livers of Nrd1+/+ mice fed the CDAA diet. n = 5–8, each. *P<0.05.
in *Nrd1*+/+ and *Nrd1*−/− fed the CSAA diet (Figure 3B), qRT-PCR showed that mRNA expression of CCR2, a recruited macrophage marker, was significantly increased in *Nrd1*+/+ mice, but not in *Nrd1*−/− mice (Figure 3C). This suggested that macrophages are not sufficiently recruited in *Nrd1*−/− mice. At 20 weeks of a CDAA feeding, production of TNF-α protein was significantly upregulated in both *Nrd1*+/+ and *Nrd1*−/− mouse livers (Figure 4A, produced TNF-α), but the increase in TNF-α protein secretion from liver specimens into the conditioned medium was decreased significantly (0.46-fold) by *Nrd1* knockout (Figure 4A, secreted TNF-α). In contrast, production of IL6 and IL1-β proteins were not increased in *Nrd1*−/− mice fed a CDAA diet (Figure 4B). These data suggested that nardilysin was required for the shedding of TNF-α in mice fed the CDAA diet and possibly the induction of inflammation. To further investigate that possibility, we examined whether blocking TNF-α secretion/production of inflammatory cytokines enhanced by nardilysin was associated with the development of liver fibrotic changes. Four weeks after CDAA feeding, fibrotic changes were not prominent in both *Nrd1*+/+ and *Nrd1*−/− mice (Figure 5A and B). Twelve weeks after CDAA feeding, fibrotic changes were observed in *Nrd1*+/+ mice, but not in *Nrd1*−/− mice. These factors were not altered by administration of the CSAA diet in *Nrd1*+/+ or *Nrd1*−/− mice. *P*<0.05.

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**Figure 6.** Fibrogenic factors were not elevated in *Nrd1*−/− mice fed the CDAA diet. During CDAA diet administration, mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA were significantly increased in the livers of *Nrd1*+/+ mice but not in those of *Nrd1*−/− mice. Those factors were not altered by administration of the CSAA diet in *Nrd1*+/+ or *Nrd1*−/− mice. *P*<0.05.

**Nrd1**−/− mice were resistant to CDAA diet-induced liver fibrotic changes

Persistent steatohepatitis results in hepatic fibrosis [1–4]. Using Sirius red staining we investigated whether secretion/production of inflammatory cytokines enhanced by nardilysin was associated with the development of liver fibrotic changes. Four weeks after CDAA feeding, fibrotic changes were not prominent in both *Nrd1*+/+ and *Nrd1*−/− mice (Figure 5A and B). Twelve weeks after CDAA feeding, fibrotic changes were observed in *Nrd1*+/+ mice,
high-power field (HPF) in livers increased only in
Nrd1−/− mice fed a CDAA diet for 20 weeks, but not in Nrd1+/+ mice. Activated myofibroblasts were hardly detected by administration of the CSAA diet in Nrd1+/+ or Nrd1−/− mice. Bars indicate 100 μm. B. The number of αSMA-positive cells/×400 high-power field (HPF) in livers increased only in Nrd1+/+ mice fed the CDAA diet for 20 weeks. *P<0.05.
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Figure 7. Activated myofibroblasts were not observed in Nrd1−/− mice fed the CDAA diet. A. Immunostainings for αSMA demonstrated that activated myofibroblasts were detected in Nrd1+/+ mice fed a CDAA diet for 20 weeks, but not in Nrd1−/− mice. Activated myofibroblasts were hardly detected by administration of the CSAA diet in Nrd1+/+ or Nrd1−/− mice. Bars indicate 100 μm. B. The number of αSMA-positive cells/×400 high-power field (HPF) in livers increased only in Nrd1+/+ mice fed the CDAA diet for 20 weeks. *P<0.05.

Nrd1−/− mice were resistant to high fat diet-induced liver fibrogenesis

To further confirm the role of nardilysin in the development of steatohepatitis followed by liver fibrotic changes, Nrd1+/+ and Nrd1−/− mice were also fed HFD. Similar to the CDAA diet, HFD administration for 20 weeks induces hepatic steatosis and liver fibrogenesis [16]. In the present study, steatosis was observed more prominently in Nrd1+/+ mice compared to Nrd1−/− mice at 20 weeks of HFD administration, but not in mice fed a normal control diet (Figure 8A). Consistently, triglyceride in the liver were elevated in Nrd1+/+ and Nrd1−/− mice (Figure 8B). However, serum ALT levels were significantly increased in Nrd1+/+ mice upon 20-week administration of the HFD, whereas they were not increased in Nrd1+/− mice compared to Nrd1−/− mice in the HFD (Figure 8C). Furthermore, fibrotic changes were detected only in Nrd1+/+ mice fed a HFD (Figure 8D and E). Consistent with this finding, qRT-PCR showed that the mRNA expression of IL1β was significantly increased only in Nrd1+/+ mice at 20 weeks of HFD feeding, but not in that of Nrd1−/− mice (Figure 9A). mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA were significantly increased in the livers of Nrd1+/+ mice fed a HFD for 20 weeks, but not in those of Nrd1−/− mice (Figure 9B). Therefore, nardilysin also played an important role in the development of steatohepatitis and liver fibrogenesis induced by HFD in mice.

Discussion

In the present study, we demonstrated that steatosis was induced by the CDAA diet in both Nrd1+/+ and Nrd1−/− mice, although fatty changes were less prominent in Nrd1−/− mice. Importantly, steatohepatitis followed by liver fibrotic changes was observed only in Nrd1+/+ mice and not in Nrd1−/− mice. Secretion of TNF-α, and the production of inflammatory cytokines and fibrogenic factors were not upregulated in Nrd1−/− mice as compared with Nrd1+/+ mice. In the HFD model, steatohepatitis and liver fibrogenesis were hardly observed in Nrd1−/− mice. These data suggested that nardilysin plays an important role in the development of steatohepatitis followed by liver fibrosis.

In mice fed with the CDAA diet, the levels of hepatic triglyceride content were lower in Nrd1−/− mice compared with those in Nrd1+/+ mice, suggesting the possibility that nardilysin is involved in the regulation of hepatic lipid synthesis. A decreased steatosis in Nrd1−/− mice may partly affect hepatic inflammation. However, steatosis did occur in the liver of Nrd1−/− mice; on the other hand, hepatic inflammation was not observed despite the presence of steatosis in Nrd1−/− mice. This indicated that nardilysin has an important role in the initiation and/or promotion of inflammatory responses induced by the CDAA diet. Persistent inflammation distinguishes steatohepatitis from simple hepatic steatosis [1–3]. Among pro-inflammatory factors, TNF-α is one of the key molecules that initiate inflammatory cascades, and its role in the progression of NASH has been discussed [4–7]. For example, apoptotic change in the liver, which contributes to the progression of NASH, is inhibited by an anti-TNF receptor neutralizing antibody or pentoxifylline in a mouse model of NASH [20]. The absence of TNFR1, a receptor for TNF-α, reduces IL6 mRNA production in the liver fed with the HFD even in the presence of elevated serum TNF-α [21]. The absence of TNFR1 also reduces liver lipid accumulation and macrophage accumulation in livers of HFD-fed mice [21]. Thus, inhibition of TNF-α signaling appears to play a pivotal role to suppress inflammatory reactions in NASH as well as other inflammatory disorders [22]. Although clinical application of anti-TNF-α therapy has not been established in the treatment of human NASH, anti-TNF-α neutralizing antibodies are effectively used to treat various human
inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel diseases [6,7]. We previously reported that nardilysin is essential for the sufficient activation of TNF-α in cooperation with TACE [10–12]. By the knockdown of Nrd1, TNF-α secretion is decreased concomitantly with decreased TACE activity, and the production of inflammatory cytokines such as IL6 and IL1-β is significantly suppressed [10–12]. In the present study, it is worth noting that TNF-α secretion from liver specimens was decreased significantly in Nrd1–/– mice fed the CDAA diet, while TNF-α production was not different between Nrd1+/+ and Nrd1–/– mice fed the CDAA diet. Consistently, the production of various inflammatory cytokines were not increased in the livers of Nrd1–/– mice. Although the precise mechanism of the decreased inflammatory responses in Nrd1–/– mice was not clear, it appeared likely that the impaired release of TNF-α in Nrd1–/– mouse livers was one of the reasons for the reduced inflammatory reactions in Nrd1–/– mice. As well, impaired recruitment of macrophages into the liver may also contribute to the reduced inflammatory reactions in Nrd1–/– mice. It would be also possible that different activation status of TNF-α and inflammatory responses conversely affect difference of fatty contents between Nrd1+/+ and Nrd1–/– mice. Whatever the case, nardilysin seemed to play an important role in the development of steatohepatitis and liver fibrosis presumably through TNF-α activation.

Previous studies have shown that Kupffer cells and recruited macrophages interact with hepatic stellate cells, accelerate their activation, and promote the fibrogenic responses [4,17]. Activated myofibroblasts also promote the remodeling of the extracellular matrix and contribute to liver fibrosis [5]. Indeed, our immunohistochemical analyses showed that Kupffer cells and macrophages were major producers of TNF-α in the livers of mice fed the CDAA diet, and that αSMA-positive myofibroblasts were not prominent in Nrd1–/– mice. Decreased release of TNF-α from Kupffer cells and recruited macrophages could be one of the mechanisms for the suppression of diet-induced steatohepatitis in Nrd1–/– mice, and thus nardilysin in Kupffer cells and recruited macrophages may be required for the progression of NASH and liver fibrosis, concomitantly with the recruitment of myofibroblasts. However, we could not completely exclude the possible
The contribution of nardilysin in other cells such as hepatocytes or endothelial cells for the development of NASH and liver fibrosis. Therefore, genetically-engineered mice lacking or strongly expressing nardilysin in Kupffer cells and macrophages may be required to confirm our hypothesis in future studies.

In summary, the present study indicates that nardilysin contributes to the development of diet-induced NASH and liver fibrotic changes by regulating chronic liver inflammation. Nardilysin could be an attractive target for anti-inflammatory therapy against NASH and liver fibrosis.

**Author Contributions**

Conceived and designed the experiments: SIH HS. Performed the experiments: SIH YM KI MT Yoshito Kimura YT Yuto Kimura YN KK. Analyzed the data: SIH HS KK. Contributed reagents/materials/analysis tools: EN HK. Wrote the paper: SIH HS TC.

**References**