(Angulo 2002; Fassio et al. 2004; Hashimoto et al. 2005). Therefore, it is important to differentiate patients with NASH from patients with more benign forms of NAFLD.

Liver fibrosis accumulates with the progression of NASH toward cirrhosis, as is reported in the case of viral hepatitis. Changes in many proteins associated with fibrosis have been reported during the course of viral hepatitis. Matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) are reportedly associated with the progression of liver fibrosis (Hemmann et al. 2007). It is unclear whether changes in the gene expression of fibrosis-associated proteins occur in the liver of patients with NASH, as they do in patients with viral hepatitis, and whether there are differences in the gene expression patterns and serum levels of these proteins between NASH and simple steatosis. In the present study, we investigated the gene expression patterns of several fibrosis-associated proteins in the livers of patients with NAFLD, comparing patients with and without NASH. Serum levels of fibrosis-associated proteins were also investigated.

Patients and methods

Patients

The study population consisted of 64 patients (36 males and 28 females with a mean age of 51.0 ± 15.0 years) who underwent ultrasound-guided liver biopsy between 2008 and 2010

for the diagnosis of NAFLD. They were patients who agreed with liver biopsy among 268 patients who had admitted to our clinics and had been advised to receive liver biopsy because of the clinical diagnosis of NAFLD during the study period. Liver biopsy was performed to examine the presence of NASH and to confirm the diagnosis. Patients were clinically diagnosed with NAFLD prior to biopsy based on the following criteria: (i) persistent abnormal liver function tests for more than 3 months, (ii) ultrasonographic images showing steatosis, (iii) no evidence of alcohol abuse, and (iv) exclusion of other liver diseases and other known causes of steatosis based on the results of specific clinical, biochemical, or imaging studies. The ultrasonographic findings of steatosis were based on established criteria such as hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring (Hamaguchi et al. 2007). The first two criteria were used as definitive criteria, while the latter two criteria were taken into account as needed. All patients were confirmed not to have chronic viral hepatitis with negative results for hepatitis B virus (HBV) surface antigen, HBV DNA, hepatitis C virus (HCV) antibody, and HCV RNA. No patients were diagnosed as having autoimmune hepatitis, primary biliary cirrhosis, or other liver diseases.

All patients underwent ultrasonography-guided fine needle liver biopsy using a 17G biopsy needle. NAFLD was pathologically diagnosed based on pathologic findings in

the biopsied liver specimens. The liver biopsy specimens were stained with hematoxylin and eosin, Masson's trichrome, and periodic-acid Schiff stains and then examined by experienced pathologists. The liver specimens were categorized into types 1–4 pathologically based on Matteoni classification (Matteoni et al. 1999), and types 3 and 4 were defined as NASH. Patologic evaluations were performed by two pathologists independently.

The study protocol was in compliance with the Helsinki Declaration and was approved by the institutional review board of Ogaki Municipal Hospital. All patients provided written informed consent for the use of their clinical data and the analyses of biopsy specimens and serum samples.

RNA extraction and real-time PCR for gene expression analyses

Liver biopsy specimens were stored in Ambion RNAlater solution (Life Technologies, Carlsbad, CA, USA) at −80°C until RNA extraction. Total RNA was extracted using the mirVana miRNA isolatidon kit (Life Technologies) according to the manufacturer's instructions.

cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 mg) in 10.4 μL of nuclease-free water was added to 1 mL of 50 mM random hexamer. The denaturing reaction was performed for 10

min at 65°C. The denatured RNA mixture was added to 4 mL of 5× reverse transcriptase buffer, 2 mL of 10 mM dNTP, 0.5 mL of 40 U/mL RNase inhibitor, and 1.1 mL of reverse transcriptase (FastStart Universal SYBR Green Master, Roche) in a total volume of 20 mL. The reaction ran for 30 min at 50°C (cDNA synthesis), and 5 min at 85°C (enzyme denaturation). All reactions were run in triplicate. The Chromo4 detector (Bio-Rad, Hercules, CA, USA) was used to detect mRNA expression. The primer sequences are follows: TIMP1: 5′-cttggcttctgcactgatgg-3′ (sense), 5′-acgctggtataaggtggtct-3′ (antisense); TIMP2: 5′-agtggactctggaaacgaca-3′ (sense); 5′-tctctgtgacccagtccatc-3′ (antisense); MMP2: 5′- aacgccgatggggagtactg-3′ (sense); 5′-cagggctgtccttcagcgtt-3′ (antisense); MMP13: 5′-gaggctccgagaaatgcagt-3′ (sense); 5′-atgccatcgtgaagtctggt-3′ (antisense); and β-actin: 5′-ccactggcatcgtgatggac-3′ (sense), 5′-tcattgccaatggtgatgacct-3′ (antisense). Assays were performed in triplicate, and the expression levels of target genes were normalized to the expression of the β-actin gene as quantified using real-time quantitative PCR as an internal control.

Measurement of serum levels of fibrosis-associated proteins

Serum levels of TIMP1, MMP2, and hyaluronic acid were measured in stored fasting serum samples that had been obtained at the time of liver biopsy. Serum TIMP1 levels were measured by enzyme immunoassay (hTIMP-1 kit, Daiichi Fine Chemical, Toyama,

Japan). Serum MMP2 levels were measured by enzyme immunoassay (hMMP-2 Activity Assay System, GE Healthcare Japan, Tokyo, Japan). Serum hyaluronic acid was measured by the latex agglutination method (Hyaluronic acid LT, Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

Quantitative values are expressed as means ± SD. Between-group differences were analyzed by the chi-square test. Differences in quantitative values between two groups were analyzed by the Mann–Whitney *U* test. Correlation between liver tissue mRNA and serum levels of TIMP1 and MMP2 were evaluated with Spearman's test. Multivariate analysis was performed using logistic regression models. All p values were 2-tailed, and $p < 0.05$ was considered to indicate statistical significance.

Results

Background characteristics of study patients

Table 1 summarizes the characteristics of the study patients. Pathologic examination revealed that all patients had steatosis involving at least 10% of the hepatocytes, and NASH was diagnosed in 43 patients (67.2%). Among 43 patients diagnosed with NASH, 13 patients (30.2%) were diagnosed with stage 3 or 4 fibrosis according to the Brunt classification (Brunt et al. 1999). No significant differences were found between patients

with and without NASH with respect to age, sex, body weight, laboratory data, and degree of steatosis on pathologic evaluation (Supplemental Table 1).

Expression of TIMP1, TIMP2, MMP2, and MMP13 mRNA in liver tissue in patients with NAFLD

Figure 1 compares the gene expression levels of TIMP1, TIMP2, MMP2, and MMP13 based on the quantification of mRNA in the liver tissue of patients with and without NASH. The quantity of MMP mRNA was significantly higher in patients with NASH (2.69 ± 1.40, relative expression level) than those without (1.50 ± 0.57; $p < 0.0001$). No significant differences were found in the quantity of TIMP1, TIMP2, and MMP13 mRNA. There were no differences in the quantity of TIMP1, TIMP2, MMP2, and MMP13 mRNA according to the degree of steatosis (data not shown).

Serum levels of TIMP1, MMP2, and hyaluronic acid in patients with NAFLD

Figure 2 compares the levels of TIMP1, MMP2, and hyaluronic acid in serum samples obtained at the time of liver biopsy between patients with and without NASH. Serum levels of TIMP1 and MMP2 showed a significant correlation with liver tissue mRNA levels, respectively, although the correlation was not strong ($p = 0.0003$ and $\rho = 0.472$ for TIMP1, and $p < 0.0001$ and $\rho = 0.534$ for MMP2, Supplemental Figure 1). The serum levels of

MMP2 and hyaluronic acid were significantly higher in patients with NASH than in patients without (MMP2, $p = 0.00198$, and hyaluronic acid, $p = 0.00042$). No significant differences were found in the serum levels of TIMP1 between patients with and without NASH.

Univariate and multivariate analyses were performed for factors associated with NASH (Table 2). In the univariate analysis, serum aspartate aminotransferase (AST), MMP2, hyaluronic acid, and type IV collagen levels were associated with NASH. In multivariate analysis, serum AST, MMP2, and hyaluronic acid levels were independently associated with NASH.

Expression of MMP2 mRNA in liver tissue and serum levels of MMP2, hyaluronic acid, and type IV collagen in NASH patients with mild fibrosis

When excluding patients with NASH and advanced fibrosis (Brunt's fibrosis stage [et al. 1999] 3 or 4), the quantity of hepatic MMP2 mRNA remained significantly higher in patients with NASH than those without ($p = 0.0010$, Figure 3). Serum levels of MMP2 were also significantly higher in patients with NASH than those without ($p = 0.0020$, Figure 3). No significant differences in serum levels hyaluronic acid were observed ($p =$

0.1296, Figure 3). In both univariate and multivariate analyses, only serum AST and MMP2 levels were associated with NASH (Table 3).

The predictive value of serum levels of MMP2 and hyaluronic acid were analyzed with receiver-operating characteristic (ROC) analysis. In all patients, serum MMP2 and hyaluronic acid levels had comparable ability for predicting NASH among patients with NAFLD with similar area under the ROC curves (AUROC) (MMP2, 0.73 and hyaluronic acid, 0.77, Supplemental Figure 2). When NASH patients with advanced fibrosis were excluded, the ability of serum hyaluronic acid levels to predict NASH decreased (AUROC, 0.63), whereas the predictive ability of serum MMP2 levels remained similar (AUROC, 0.74, Supplemental Figure 3).

Discussion

In the present study, we observed enhanced gene expression of MMP2 in liver tissue, along with elevated serum levels of MMP2, both of which showed the correlation, in patients with NASH compared to those with simple steatosis. MMP2 expression reportedly increases during the progression of liver fibrosis (Ebata et al. 1997; Hemmann et al. 2007), and our results indicated that liver fibrosis is proceeding in patients with NASH.

NASH is usually diagnosed by histological evaluation of specimens obtained by liver biopsy and this is currently the only reliable and accepted method for the evaluation of

liver fibrosis. However, liver biopsy is invasive and carries the risk of intraperitoneal bleeding. Therefore, noninvasive indicators of NASH in NAFLD patients would be important. Several biomarkers have been studied as indicators of NASH in patients with NAFLD (Malik et al. 2009; Miele et al. 2009). The serum level of hyaluronic acid is an important marker for the identification of patients with NASH. Because serum hyaluronic acid levels increase with the progression of liver fibrosis (Adams 2011) and the increase can simply reflect accumulated fibrosis in the liver as a result of the progression of NASH, it may not be an indicator of NASH but with mild fibrosis (i.e. early stage of NASH). Although serum hyaluronic acid levels were significantly higher in patients with NASH than those without NASH in the present study, we failed to find significant differences in serum hyaluronic acid levels between NASH patients with mild fibrosis and those without NASH. Whereas the AUROC for serum hyaluronic acid level in predicting NASH was more than 0.7 in patients including advanced fibrosis in the study by Malik et al. and in the present study, it decreased from 0.77 to 0.63 when focusing on patients with mild fibrosis in the present study. Serum hyaluronic acid levels, therefore, do not appear to be useful for distinguishing NASH patients with mild fibrosis from patients without NASH. In contrast, the AUROC for serum MMP2 levels in predicting NASH remains greater than 0.73 even in the subpopulation with mild fibrosis. In clinical practice, it will be important to identify

NASH patients with mild fibrosis, before the progression of liver fibrosis caused by NASH, from NAFLD patients. Serum MMP2 levels may be useful for this purpose.

Conclusion

In patients with NASH, gene expression of MMP2, a protein associated with liver fibrosis, was enhanced in the liver tissue and serum levels of MMP2 were increased, indicating the initiation of liver fibrosis in this subpopulation. These results were also observed when NASH patients with advanced fibrosis were excluded. MMP2 may be a noninvasive indicator of early stage of NASH in patients with NAFLD.

Declaration of interest

The authors declare no conflicts of interest.

References

Adams LA. (2011). Biomarkers of liver fibrosis. *J Gastroenterol Hepatol* 26:802–809.

Angulo P. (2002). Nonalcoholic fatty liver disease. *N Engl J Med* 346:1221–1231.

Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. (1999). Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions. *Am J Gastroenterol* 94:2467–2474.

Chitturi S, Farrell GC, Hashimoto E, Saibara T, Lau GK, Sollano JD; Asia-Pacific Working Party on NAFLD. (2007). Non-alcoholic fatty liver disease in the

Asia-Pacific region: Definitions and overview of proposed guidelines. *J Gastroenterol Hepatol* 22:778–787.

Clark JM, Brancati FL, Diehl AM. (2003). The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol* 98:960–967.

Ebata M, Fukuda Y, Nakano I, Katano Y, Fujimoto N, Hayakawa T. (1997). Serum levels of tissue inhibitor of metalloproteinases-2 and of precursor form of matrix metalloproteinase-2 in patients with liver disease. *Liver* 17:293–299.

Fassio E, Alvarez E, Domínguez N, Landeira G, Longo C. (2004). Natural history of nonalcoholic steatohepatitis: A longitudinal study of repeat liver biopsies. *Hepatology* 40:820–826.

Hamaguchi M, Kojima T, Itoh Y, Harano Y, Fujii K, Nakajima T, Kato T, Takeda N, Okuda J, Ida K, Kawahito Y, Yoshikawa T, Okanoue T. (2007). The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation. *Am J Gastroenterol* 102:2708–2715.

Hashimoto E, Yatsuji S, Kaneda H, Yoshioka Y, Taniai M, Tokushige K, Shiratori K. (2005). The characteristics and natural history of Japanese patients with nonalcoholic fatty liver disease. *Hepatol Res* 33:72–76.

Hemmann S, Graf J, Roderfeld M, Roeb E. (2007). Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies. *J Hepatol* 46:955–975.

Kojima S, Watanabe N, Numata M, Ogawa T, Matsuzaki S. (2003). Increase in the prevalence of fatty liver in Japan over the past 12 years: Analysis of clinical background. *J Gastroenterol* 38:954–961.

Malik R, Chang M, Bhaskar K, Nasser I, Curry M, Schuppan D, Byrnes V, Afdhal N. (2009). The clinical utility of biomarkers and the nonalcoholic steatohepatitis CRN liver biopsy scoring system in patients with nonalcoholic fatty liver disease. *J Gastroenterol Hepatol* 24:564–568.

Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. (1999). Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity. *Gastroenterology* 116:1413–1419.

Miele L, Forgione A, La Torre G, Vero V, Cefalo C, Racco S, Vellone VG, Vecchio FM, Gasbarrini G, Rapaccini GL, Neuman MG, Grieco A. (2009). Serum levels of hyaluronic acid and tissue metalloproteinase inhibitor-1 combined with age predict the presence of nonalcoholic steatohepatitis in a pilot cohort of subjects with nonalcoholic fatty liver disease. *Transl Res* 154:194–201.

Musso G, Gambino R, Cassader M. (2010). Non-alcoholic fatty liver disease from pathogenesis to management: An update. *Obes Rev* 11:430–445.

Torres DM, Harrison SA. (2008). Diagnosis and therapy of nonalcoholic steatohepatitis. *Gastroenterology* 134:1682–1698.

Table 1. Characteristics of study patients ($n = 64$).

Age (years)	51.0 ± 15.0
Sex (male/female)	36 (56.3)/28 (43.7)
Body weight (kg)	70.3 ± 11.5
Body mass index (kg/m^2)	27.1 ± 3.5
Alanine aminotransferase (IU/L)	88.5 ± 76.4
Aspartate aminotransferase (IU/L)	54.3 ± 32.5
Gamma-glutamyl transpeptidase (IU)	87.1 ± 64.6
Total bilirubin (mg/dL)	0.71 ± 0.54
Albumin (g/dL)	4.22 ± 0.51
Glucose (mg/dL)*	131.6 ± 61.3
Total cholesterol (mg/dL)*	197.8 ± 39.4
Triglyceride (mg/dL)*	162.1 ± 84.3
Hemoglobin A_1-c (%)	6.07 ± 1.55
Hemoglobin (g/dL)	14.7 ± 1.6
Platelet count (×10^3/μL)	238 ± 70
Ferritin (ng/mL)	231.0 ± 190.7
Steatosis (<30%/30–50%/50–70%/70%≤)**	17 (26.6)/18 (28.1)/19 (29.7)/10 (15.6)

Diagnosis (NASH/simple steatosis)	43 (67.2)/21 (32.8)
Fibrosis (grade 1/2/3/4)**,#	20 (46.5)/10 (23.3)/10 (23.3)/3 (6.9)

NASH, non-alcoholic steatohepatitis.

*Measured under fasting conditions.

**Based on pathologic examination.

#Only in patients with NASH by Brunt classification (Brunt 1999).

Table 2. Univariate and multivariate analyses for distinguishing between patients with NASH and simple steatosis (n = 64).

	Univariate analysis (p value)	Multivariate analysis (p value)	Odds ratio (95% confidence interval)
Age (years)	0.4523	–	
Sex (male/female)	0.9199	–	
Body weight (kg)	0.4524	–	
Body mass index (kg/m^2)	0.2999	–	
Alanine aminotransferase (IU/L)	0.2061	–	
Aspartate aminotransferase (IU/L)	0.0146	0.0258	938.371 (4.9097–922101.2)
Gamma-glutamyl transpeptidase (IU)	0.5724	–	
Total bilirubin (mg/dL)	0.1126	–	
Albumin (g/dL)	0.1959	–	
Glucose (mg/dL)*	0.1898	–	

Total cholesterol (mg/dL)*	0.2338	–	
Triglyceride (mg/dL)*	0.1696	–	
Hemoglobin A_1-c (%)	0.9370	–	
Hemoglobin (g/dL)	0.5974	–	
Platelet count ($\times 10^3/\mu L$)	0.0613	–	
Ferritin (ng/mL)	0.5443	–	
TIMP1 (ng/mL)	0.2224	–	
MMP2 (ng/mL)	0.0058	0.0275	364.171 (3.9968–174225.9)
Hyaluronic acid (ng/mL)	0.0228	0.0351	23346.68 (32.5694–298598)
Steatosis (<30%/30–50%/50–70%/70%≤)**	0.8713	–	

NASH, non-alcoholic steatohepatitis; TIMP1, tissue inhibitor metalloproteinase-1; MMP2, matrix metalloproteinase-2.

*Measured during fasting conditions.

**Based on pathologic examination.

Table 3. Univariate and multivariate analyses for distinguishing patients with NASH simple steatosis, excluding NASH patients with advanced fibrosis ($n = 51$).

	Univariate analysis (p value)	Multivariate analysis (p value)	Odds ratio (95% confidence interval)
Age (years)	0.7699	–	
Sex (male/female)	0.9730	–	
Body weight (kg)	0.3069	–	

Body mass index (kg/m^2)	0.2221	–	
Alanine aminotransferase (IU/L)	0.1769	–	
Aspartate aminotransferase (IU/L)	0.0309	0.0251	240.057 (3.5678–72252.5)
Gamma-glutamyl transpeptidase (IU)	0.7185	–	
Total bilirubin (mg/dL)	0.1344	–	
Albumin (g/dL)	0.3718	–	
Glucose (mg/dL)*	0.5584	–	
Total cholesterol (mg/dL)*	0.5173	–	
Triglyceride (mg/dL)*	0.1327	–	
Hemoglobin A_1-c (%)	0.7368	–	
Hemoglobin (g/dL)	0.8608	–	
Platelet count ($\times 10^3/\mu L$)	0.5635	–	
Ferritin (ng/mL)	0.8109	–	
TIMP1 (ng/mL)	0.2302	–	
MMP2 (ng/mL)	0.0047	0.0068	2759.72 (19.7697–2163013)
Hyaluronic acid (ng/mL)	0.1007	–	
Steatosis (<30%/30–50%/50–70%/70%≤)**	0.2877	–	

NASH, non-alcoholic steatohepatitis; TIMP1, tissue inhibitor metalloproteinase-1; MMP2, matrix metalloproteinase-2.

*Measured during fasting conditions.

**Based on pathologic examination.

Figure 1. Relative mRNA expression levels of tissue inhibitor metalloproteinase-1 and -2 (TIMP1 and TIMP2), and matrix metalloproteinase 2 and 13 (MMP2 and MMP13) in the liver tissue of patients with nonalcoholic steatohepatitis (NASH) versus simple steatosis.

Figure 2. Serum levels of tissue inhibitor metalloproteinase-1 (TIMP1), matrix metalloproteinase-2 (MMP2), and hyaluronic acid in all patients with nonalcoholic steatohepatitis (NASH) versus simple steatosis. TIMP1, 277.4 ± 64.6 ng/mL with NASH vs. 256.5 ± 55.6 ng/mL with simple steatosis; $p = 0.2906$, MMP2, 427.8 ± 95.2 ng/mL with NASH vs. 353.6 ± 55.1 ng/mL with simple steatosis; $p = 0.0198$, and hyaluronic acid, 111.0 ± 131.5 ng/mL with NASH vs. 29.3 ± 21.2 ng/mL with simple steatosis; $p = 0.00042$.

Figure 3. Quantity of matrix metalloproteinase-2 (MMP2) mRNA in liver tissue and serum levels of MMP2, hyaluronic acid, and type IV collagen in nonalcoholic steatohepatitis (NASH) patients without advanced fibrosis versus patients with simple steatosis. MMP2 mRNA, 2.23 ± 0.84 with NASH vs. 1.50 ± 0.57 with simple steatosis; $p = 0.0010$, serum levels of MMP2, 441.8 ± 99.6 ng/mL with NASH vs. 353.6 ± 55.1 ng/mL with simple steatosis; $p = 0.0020$, and serum levels of hyaluronic acid, 143.1 ± 31.1 ng/mL with NASH vs. 130.3 ± 63.1 ng/mL with simple steatosis; $p = 0.1296$.

タイトル：ウイルス感染と分泌型 microRNA

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重要なポイント

・エクソソームは直径 30-100nm の脂質二重膜構造を有した顆粒であり、分泌細胞由来の microRNA を含有している。

・血中エクソソーム中の microRNA を解析することにより、解析対象臓器の生検をせずに特異度の高い疾患の診断や病態を把握することができる。

・エクソソームは細胞間情報伝達に深く関わっており、肝炎ウイルス感染肝細胞から非感染肝細胞へのウイルス粒子の伝播にエクソソームが関与している可能性がある。

・はじめに

近年採血等患者の侵襲の少ない方法で疾患の診断を行う試みがなされている。特に体液中に存在する遺伝子の発現パターンを捉えることで、従来の検査では診断が困難であった疾患の診断が可能であることがわかり、その有用性に期待が寄せられている。

本稿ではエクソソーム中の microRNA(miRNA)の発現解析をすることによってウイルス性肝炎や脂肪肝を含めた慢性肝疾患の診断を行う方法、ウイルス感染が惹起するエクソソーム中の miRNA 発現に対する影響について近年の知見を示し、エクソソーム/miRNA を用いたウイルス性肝炎に対する新規核酸治療の可能性について概説する。

・miRNA とは

miRNA は、塩基配列特異的に標的遺伝子の 3'非翻訳領域(UTR)に結合し、標的遺伝子の翻訳を阻害することにより遺伝子発現を制御している 20 塩基長前後の機能的小分子 RNA であり、生命現象の根幹である発生や分化誘導だけでなく、発癌や各種変性疾患に関与していることが報告されている [1]。現在、ヒトにおいては 1872 種の前駆体 miRNA(precursor miRNA)と 2578 種の成熟体 miRNA(mature miRNA)の存在が報告されている[(miRBase ver.20 (http://www.mirbase.org/index.shtml)]。miRNA の特徴として、個体間では発現量の差が少ないが、臓器や細胞ごとに異なる発現プロファイルを有している。このことから miRNA による遺伝子制御は各臓器の恒常性を保つ役割の一端をなしていると考えられている。肝組織で発現している miRNA 中約 70%が miR-122 であり [2]、肝臓において miR-122 の発現の程度は、コレステロール合成、成人の肝機能の維持、日内変動に関係する遺伝子の調整による肝機能の維持、さらに慢性肝疾患の進展、特に発癌、線維化、治療効果などと深く関係しているとの報告がある (図 1)。

・エクソソームとは

エクソソームは直径 30-100nm の脂質二重膜を有する小粒子であり、末梢血のみならず、あらゆる体液中に存在する。エクソソームは、元々は細胞内の老廃物を細胞外に放出するものとして捉えられていたが、生合成過程において宿主細胞に由来する細胞質に存在するサイトカイン、メッセンジャーRNA(mRNA)や miRNA がエクソソーム内に取り込まれることが報告され [3]、これらの分子が細胞間の情報伝達に関与していることが考えられている。また、体液中のエクソソームが内包する miRNA のプロファイルが各種疾患の病態に伴い変化することが知られており、各疾患の診断ツールとしても注目されている(図 2)。

・末梢血中エクソソームを用いた慢性肝疾患の診断

末梢血中のエクソソームを用いた慢性肝疾患の診断に関する報告例を表 1 で示した。現在、慢性肝疾患の診断には血清あるいは血漿に含まれる total RNA を抽出し、miRNA の解析を行う方法が主流であるが、筆者らはエクソソーム中の miRNA を解析対象とすることにより、

より再現性の高い解析結果を得ることができ、エクソソームが疾患特異度の高いバイオマーカーとして有用である[4]ことを示した。

エクソソームの解析において、現時点ではエクソソームのみを単離することは困難であるため、エクソソームが豊富に含まれる画分を抽出して、それをエクソソーム画分として解析がなされている(以後エクソソーム画分という)。通常エクソソーム画分を採取するには古典的には超遠心法、最近では polyethylene glycol を使った凝集方法がキット化されて広く使われている。今回我々はベッドサイドで容易に行うことができる ExoQuick®(System Bioscience)を用いて解析を行った。ExoQuick®で単離したエクソソーム画分にはエクソソーム表面に発現しているマーカーの一つである CD63 のタンパクの発現が確認でき 、この画分にエクソソームが含まれていることを確認した。

miRNA の発現の再現性を確認するため、同一検体から得られた血清を用いて、血清と Exoquick®で単離したエクソソーム画分からそれぞれ 2 回ずつ total RNA を抽出し、合計 4 検体に対してマイクロアレイ(Agilent human miRNA microarray Rel 14.0)を用い miRNA 発現解析を行った。それぞれの検体から得られたマイクロアレイ結果を比較した所、エクソソーム画分にて解析を行った場合の miRNA 発現の相関係数は 0.998 (pearson's correlation)であった。血清で行った場合の相関係数は 0.930 であり、解析の再現性はエクソソーム画分を使った方が良好であった。この結果に基づき、正常肝患者(NL)24 例、慢性 C 型肝炎患者(CHC)64 例の血清から Exoquick®で単離したエクソソーム画分を用いて両者の分別を試みた。9 種類の miRNA の発現パターンを LOOCV(leave one out cross-validation)[5]により分類したところ、96.6%の accuracy と AUC 0.98 が得られた(図 3a,b)。さらに、NL24 症例と慢性 B 型肝炎(CHB)4 症例では 89.3%の accuracy と 0.92 の AUC を (図 3c,d)、さらに、NL24 症例と非アルコール性脂肪性肝炎(NASH) 12 症例では 88.2%の accuracy と 0.82 の AUC が得られた(図 3e.f)。

また、CHC における肝の炎症と線維化レベルについてあらかじめ肝生検組織について Metavir 分類を用いて診断を行い、miRNA の発現プロファイルと比較した。肝の炎症の評価を LOOCV にて A1 と A0+A2+A3 の両群に分別し、同様に A2 と A0+A1+A3, A3 と A0+A1+A2 に分類した所 accuracy はそれぞれ 71.9%, 75.0%, 82.8%であり、オッズ比はそれぞれ 7.08, 9.50, 11.08 であった(A0 は 1 症例であったため A0 のみの分別は行っていない)。肝線維化に対する評価を同様に LOOCV にて F0 と F1+F2+F3 に分別し、同様に F1 と F0+F2+F3、F2 と F0+F1+F3、F3 と F0+F1+F2 に分類した所 accuracy はそれぞれ 89.1%, 67.2%, 73.4%, 73.4%であり、オッズ比はそれぞれ 16.71, 4.12, 7.77, 7.84 であった。

・ウイルス性肝炎の病態とエクソソームの関連性

近年、HCV 感染肝細胞から非感染肝細胞に、エクソソームを介してウイルス粒子の伝達が行われている可能性があることが報告されている[6]。また、肝炎ウイルス感染肝細胞から分泌されるエクソソームが形質細胞様樹状細胞(pDC)に取り込まれ、インターフェロン(IFN)