contained fat droplets. The histologic quantification of hepatic iron was done according to Deugnier et al. (17) by scoring iron separately within hepatocytes (hepatic iron score, 0-36), sinusoidal cells (sinusoidal iron score, 0-12), and portal tracts or fibrotic tissue (portal iron score, 0-12). The total iron score (TIS, 0-60) was defined by the sum of these scores. This score has been shown to highly correlate with the biochemical hepatic iron index and hepatic iron concentration as measured by the atomic absorption spectrophotometry in patients with chronic liver diseases (18-20). All histologic grading and staging were done by a single pathologist without knowledge of the patients' clinical and laboratory data.

Immunohistochemical Detection of 8-oxodG Adducts in Liver Biopsy Samples. Immunohistochemical staining of 8-oxodG was done as previously described (21). Mouse monoclonal antibody against 8-oxodG (Japan Institute for the Control of Aging, Shizuoka, Japan) and Alexa Fluor 488–labeled goat antibody against mouse IgG (Molecular Probes) were used. The degree of immunoreactivity was estimated by counting the number of stained hepatocyte nuclei using Adobe Photoshop version 5.5 and NIH Image free software (version 1.62, NIH, Image program; ref. 21).

The specificity of the anti-8-oxodG antibody used in this study was confirmed by several parallel experiments. Sections in which the primary antibody was omitted or those treated with normal control serum instead of the primary 8-oxodG antibody consistently yielded negative staining. Localization of 8-oxodG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with 25 ng/mL of 8-oxodG but not by over a thousand-fold greater concentration of guanosine. When the primary antibody was preincubated with graded 8-oxodG competitively, a similar blocking of immunolabeling was obtained. Further, enzymatic treatment with RNase did not affect the immunoreactivity toward oxidized DNA.

Iron Reduction Therapy for NASH. To evaluate the clinical effects of iron reduction for NASH, 11 NASH patients with iron overload [serum ferritin levels were elevated above the reference range (>300 ng/mL for male and >200 ng/mL for female)] underwent iron reduction therapy and the changes of serum and histologic features were analyzed. We selected patients that fulfilled the following criteria for iron reduction: no complication with hypertension and/or cardiovascular disorder, <70 y, and their histology showed without cirrhosis and TIS is not score 0. Iron depletion was accomplished by doing intermittent phlebotomies in combination with regulation of dietary iron intake as described previously (22). In brief, at the initial phase of iron depletion, all patients underwent weekly or biweekly phlebotomy of 200 g until a state of mild iron deficiency was achieved (defined by a serum ferritin levels <50 ng/mL and/or a blood hemoglobin concentration of 12 g/dL). The mild iron deficiency state was maintained by additional phlebotomies during the study period: patients were followed up every 1 to 2 mo for the duration, and a phlebotomy was done if the serum ferritin level exceeded 80 ng/mL. In addition, those subjects were instructed both orally and in writing by a registered dietitian to reduce their intake of iron-rich foods during the intervention. The subjects were not required to alter their total caloric intake but were expected to replace iron-rich foods with appropriate substitutes.

Statistical Analysis. Results are presented as the medians and ranges for quantitative data or as numbers with percentages in parentheses for qualitative data. Demographic and baseline data were compared

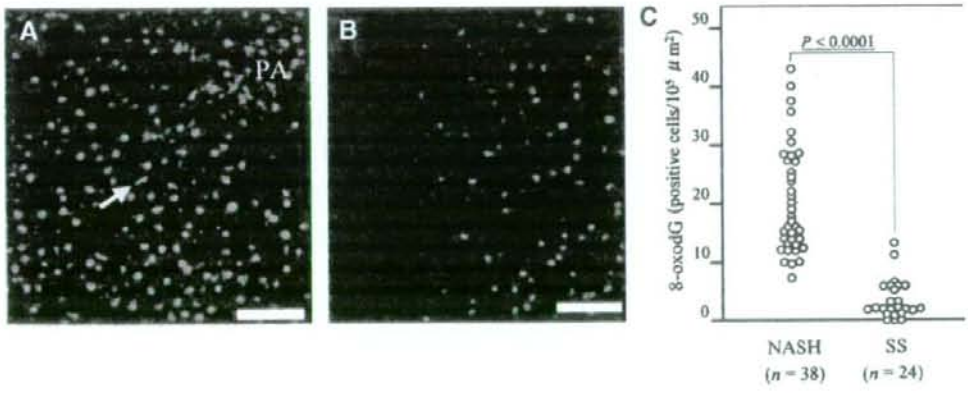

Figure 1. A and **B.** Representative 8-oxodG immunohistochemical staining in liver tissues from patients with NASH (**A**) and simple steatosis (**B**). In the liver of NASH, 8-oxodG immunoreactivity was strongly observed at the nuclei of many hepatocytes and several Kupffer cells (*arrow*) throughout the whole acinus. *PA*, portal area. In the liver of simple steatosis, relatively faint immunoreactivity of 8-oxodG was observed in the nuclei of hepatocytes and rarely in the cytoplasm. *Scale bar*, 100 μm. **C.** Comparison between 8-oxodG–positive hepatocytic nuclear counts of patients with NASH and those of simple steatosis (*SS*). Positive cells were significantly higher in NASH patients than in simple steatosis. O, individual data of patients.

Table 2. Correlations between clinical findings and 8-oxodG levels in the liver of patients with NASH (n = 38)

Characteristics	8-oxodG	Statistics	
	(positive cells/10^5 μm^2)	r	P
Age (y)		0.048*	NS*
Gender			
Male (n = 22)	20.7 (10.0-43.3)†		NS‡
Female (n = 16)	15.4 (7.3-35.7)†		
BMI (kg/m^2)		−0.057*	NS*
Laboratory data			
ALT (IU/L)		−0.012*	NS*
AST (IU/L)		0.068*	NS*
Total cholesterol (mg/dL)		−0.258*	NS*
Triglyceride (mg/dL)		−0.050*	NS*
Glucose (mg/dL)		0.628*	0.0001*
Serum insulin (microunits/mL)		0.359*	0.0294*
HOMA-IR		0.683*	<0.0001*
Hyaluronic acid (ng/mL)		0.307*	NS*
Platelet count ($\times 10^4$/mm^3)		−0.491*	0.0028*
RBC count ($\times 10^4$/mm^3)		−0.119*	NS*
Hemoglobin (g/dL)		0.009*	NS*
Serum iron (μg/dL)		0.587*	0.0004*
Transferrin saturation (%)		0.364*	0.0267*
Serum ferritin (ng/mL)		0.325*	0.0481*
Liver histology			
Inflammatory activity§			
A1 (n = 14)	18.9 (10.0-40.0)†		
A2 (n = 21)	19.0 (7.3-43.3)†		NS‖
A3 (n = 3)	14.7 (12.0-17.6)†		
Fibrosis staging§			
F1 (n = 8)	14.9 (10.0-43.3)†		
F2 (n = 17)	15.0 (7.3-37.7)†		NS‖
F3/4 (n = 13)	21.0 (12.0-40.0)†		
Steatosis¶		0.392*	0.0172*
TIS**		0.455*	0.0056*

*Spearman rank correlation test.
†Data are expressed as median (range).
‡Unpaired Student's t test.
§Inflammatory activity and fibrosis staging in NASH was scored according to Brunt classification (16).
‖One-way factorial ANOVA and multiple comparison test.
¶Hepatic steatosis degree was assessed based on the percentage of affected hepatocytes.
**The histologic quantification of iron was assessed by TIS proposed by Deugnier et al. (17).

by use of Kruskal-Wallis ANOVA, which is independent of the distribution of the data. Distribution of variables was first evaluated to determine the most appropriate statistical method across group comparisons. Normally distributed data were compared using one-way ANOVA. Data that were not normally distributed were analyzed using Kruskal-Wallis ANOVA. The mean values of two groups of normally distributed data were compared by a t test, and the median values of two groups of data that were not normally distributed were compared using the Mann-Whitney U test. Spearman rank correlation was used to quantify the association between continuous or ordered categorical variables. To analyze the changes of BMI, serum, and histologic variables after the iron reduction therapy, paired Student's t test was used. Logistic regression analysis was used to identify significant factors that influence elevated hepatic 8-oxodG expression in NASH and simple steatosis patients. Categorical variables with more than two levels were coded as dummy variables. All tests were two tailed, and P values <0.05 were considered as statistically significant. Statistical analysis was done using the commercially available software Statistical Package for the Social Sciences 11.5 (SPSS, Inc.).

Results

Clinical Characteristics of the Patients with NASH and Simple Steatosis. The main demographic and clinical laboratory features of the patients with NASH and simple steatosis are compared in Table 1. Patients with NASH were older, and more male and obese subjects than in simple steatosis, but they did not reach the statistical significance. The prevalence of type II diabetes, hypertension, and hyperlipidemia, and serum total cholesterol, triglyceride, and glucose levels were not significantly different between the two groups. Serum aspartate aminotransferase (AST), fasting insulin levels, insulin resistance [assessed by homeostasis model assessment of insulin resistance (HOMA-IR)], and hyaluronic acid were significantly higher in NASH than in simple steatosis. Iron-related serum markers (i.e., serum iron, transferrin saturation, and ferritin) were found to be significantly elevated in NASH compared with those of simple steatosis. Although liver histology showed no significant difference in steatosis degree between the NASH and simple steatosis, hepatic iron deposition was more prominent in NASH; TIS was significantly higher in NASH compared with simple steatosis [3 (0-8) versus 0 (0-5); $P < 0.0001$].

Hepatic 8-oxodG Levels in NASH and Simple Steatosis Patients. Figure 1A and B showed the 8-oxodG immunohistochemical staining in liver biopsy samples in patients with NASH and simple steatosis, as representative. 8-oxodG immunoreactivity was strongly observed in the nuclei (and weakly in the cytoplasm) of hepatocytes, Kupffer cells, and infiltrated inflammatory cells in NASH patients' liver biopsy specimen (Fig. 1A). The hepatocyte nuclei were differentiated from the nuclei of other cells using computed analyses at the point of nuclear shape and size. 8-oxodG–immunoreactive hepatocytes were distributed throughout the whole acinus in liver of patients. Using the liver samples of patients with simple steatosis, relatively faint immunoreactivity of 8-oxodG was observed in the nuclei of hepatocytes and was rarely in the cytoplasm (Fig. 1B). As a whole, 8-oxodG–positive hepatocyte counts were significantly higher in NASH patients than in simple steatosis [17.5 (range, 7.3-43.3) versus 2.0 (range, 0.0-13.3) cells/10^5 μm^2; $P < 0.0001$; Fig. 1C]. In the liver of 10 healthy controls, immunoreactivities of 8-oxodG were rarely detected in the nuclei of hepatocytes.

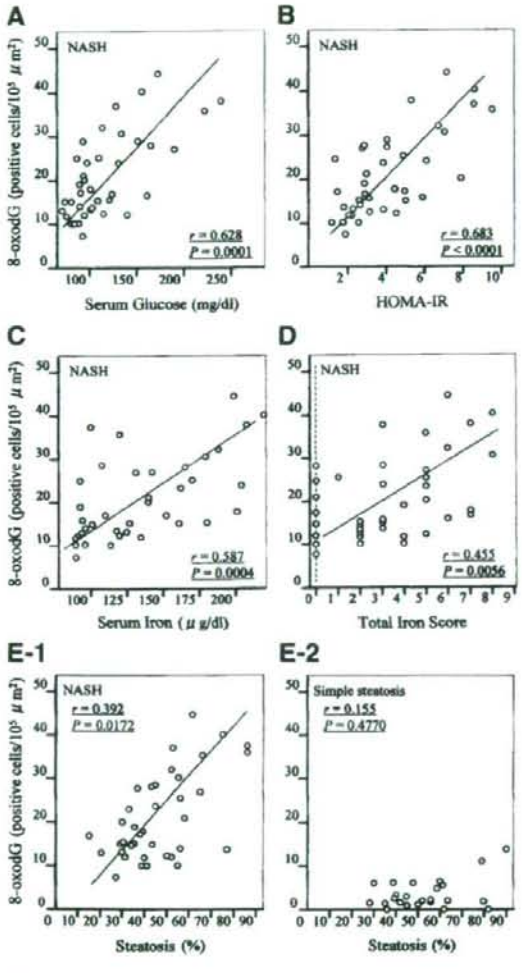

Figure 2. Correlations between 8-oxodG–positive hepatocytic nuclear counts and clinical variables in 38 NASH or 24 simple steatosis patients. **A.** 8-oxodG counts and serum glucose levels in NASH. **B.** 8-oxodG counts and HOMA-IR in NASH. **C.** 8-oxodG counts and serum iron levels in NASH. **D.** 8-oxodG counts and TIS in hepatic tissues in NASH. Dotted vertical line indicates that the TIS is 0. **E-1.** 8-oxodG counts and extent of hepatic steatosis in NASH. **E-2.** 8-oxodG counts and extent of hepatic steatosis in simple steatosis.

Clinical Variables That Correlate with Hepatic 8-oxodG Levels in NASH Patients. To estimate the source of oxidant-generated DNA damage that frequently occurred in the livers of patients with NASH, the correlations of clinical and histologic findings with the degree of hepatic damaged DNA were evaluated, and the results are summarized in Table 2. Patients' age, gender, and BMI were not related to hepatic 8-oxodG counts in NASH patients. Although the 8-oxodG–positive hepatocytic counts were not correlated with serum transaminases, cholesterol, and triglyceride levels, hepatic 8-oxodG levels were elevated in parallel with increase of fasting glucose, serum insulin, and HOMA-IR in patients with NASH [8-oxodG versus glucose ($r = 0.628$, $P = 0.0001$) versus serum insulin ($r = 0.359$, $P = 0.0294$) versus HOMA-IR ($r = 0.683$, $P < 0.0001$); Table 2; Fig. 2A and B]. It is noteworthy that the hepatic 8-oxodG levels were also positively correlated with body and hepatic iron deposition markers; serum iron, transferrin saturation, ferritin, and the hepatic iron deposit grade (i.e., TIS) were significantly correlated with 8-oxodG–positive hepatocyte nucleus counts [8-oxodG versus iron ($r = 0.587$, $P = 0.0004$) versus transferrin saturation ($r = 0.364$, $P = 0.0267$) versus ferritin ($r = 0.325$, $P = 0.0481$) versus TIS ($r = 0.455$, $P = 0.0056$); Table 2; Fig. 2C and D]. Platelet count was also correlated with hepatic 8-oxodG levels, but histologic features (inflammatory activity and fibrosis staging) were not related to hepatic oxidative damage to DNA in patients with NASH. Moreover, elevated hepatocytic 8-oxodG levels were significantly correlated with the extent of hepatic steatosis in patients with NASH (8-oxodG versus steatosis, $r = 0.392$, $P = 0.0172$; Fig. 2E-1), but these two variables were not related in patients with simple steatosis (Fig. 2E-2). The degree of hepatic iron deposition (TIS) and insulin resistance (HOMA-IR) was also correlated mutually in patients with NASH (Fig. 3).

Clinical Variables That Correlate with Hepatic 8-oxodG Levels in Simple Steatosis Patients. The correlations of clinical and histologic findings with the hepatic 8-oxodG levels were also investigated in simple steatosis patients (Table 3). Patients' age and serum ferritin levels were significantly related to hepatic 8-oxodG levels in simple steatosis, but other variables, including HOMA-IR, serum iron levels, and TIS, were not correlated.

Factors Independently Associated with Elevated Hepatic 8-oxodG Levels. To identify the variables independently associated with elevated hepatic 8-oxodG

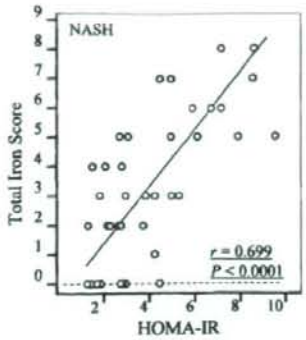

Figure 3. Correlation between TIS in hepatic tissues and HOMA-IR in NASH patients. Dotted horizontal line indicates that the TIS is 0.

levels in NASH and simple steatosis patients, logistic regression analysis was done using the variables recorded in Tables 2 and 3. When the analysis was done in combination NASH and simple steatosis, positive for hepatic iron deposit (i.e., TIS > 0) and insulin resistance (i.e., HOMA-IR > 2) were independent variables contributing to elevated (>10 positive cells/10^5 μm^2) hepatic 8-oxodG (Table 4).

Changes of Serum and Hepatic Histologic Features by Iron Reduction in NASH Patients. To directly evaluate the effect of iron overload to oxidatively generated damage to DNA in the liver of patients with NASH, iron reduction therapy (phlebotomy plus iron-restricted diet) was done in 11 hyperferritinemic NASH patients (7 males and 4 females; range, 39-67 years) and changes of serum and histologic variables were examined (Table 5). A mean blood volume of 1,700 ± 630 mL was removed by 8.5 ± 3.1 venesection times done over a period of 10.8 ± 1.9 months. Serum hemoglobin, iron, and ferritin levels were decreased in all treated patients at the end of iron reduction. Serum alanine aminotransferase (ALT), TIS score, and hepatic 8-oxodG levels were also decreased in most treated patients, and mean values were significantly decreased after the treatment. Serum cholesterol, triglyceride, fasting glucose, and insulin levels were not significantly changed by iron reduction therapy.

Discussion

In this study, we used immunohistochemical approaches using a monoclonal antibody against 8-oxodG in formalin-fixed, paraffin-embedded liver sections for assessment of oxidatively generated damage to DNA in the liver of nonalcoholic fatty liver disease. Using this approach, 8-oxodG-positive signals in liver tissue were detected in all patients with NASH, suggesting that oxidative stress is a frequent event in the liver of NASH patients. At present, a commonly accepted model for the pathogenesis of NASH is the so-called two-hit hypothesis; first hit leads to accumulation of hepatic free fatty acids resulting in a histologic picture of macrovesicular steatosis, and a subsequent second hit may result in liver

Table 3. Correlations between clinical findings and 8-oxodG levels in the liver of patients with simple steatosis (n = 24)

Characteristics	8-oxodG (positive cells/10^5 μm^2)	Statistics	
		r	P
Age (y)		0.485*	0.0251*
Gender			
Male (n = 11)	2.0 (0.7-13.3)†		NS‡
Female (n = 13)	2.0 (0.0-6.3)†		
BMI (kg/m^2)		0.221*	NS*
Laboratory data			
ALT (IU/L)		0.276*	NS*
AST (IU/L)		0.310*	NS*
Total cholesterol (mg/dL)		0.009*	NS*
Triglyceride (mg/dL)		−0.070*	NS*
Glucose (mg/dL)		0.321*	NS*
Serum insulin (microunits/mL)		−0.225*	NS*
HOMA-IR		0.001*	NS*
Hyaluronic acid (ng/mL)		0.360*	NS*
Platelet count (×10^4/mm^3)		−0.265*	NS*
RBC count (×10^4/mm^3)		−0.265*	NS*
Hemoglobin (g/dL)		−0.237*	NS*
Serum iron (μg/dL)		0.094*	NS*
Transferrin saturation (%)		0.141*	NS*
Serum ferritin (ng/mL)		0.577*	0.0082*
Liver histology			
Steatosis§		0.155*	NS*
TIS‖		0.282*	NS*

*Spearman rank correlation test.
† Data are expressed as median (range).
‡ Unpaired Student's t test.
§ Hepatic steatosis degree was assessed based on the percentage of affected hepatocytes.
‖ The histologic quantification of iron was assessed by TIS proposed by Deugnier et al. (17).

Table 4. Factors associated with the elevated hepatic 8-oxodG in NASH and simple steatosis patients by regression analysis

Factors	RR (95% CI)	*P*
TIS > 0	3.69 (2.18-13.97)	0.0088
HOMA-IR > 2	2.61 (1.50-6.46)	0.0273

Abbreviations: RR, relative risk; 95% CI, confidence interval.

injury (3). Although the precise mechanism of how the second hit occurs and concerns in liver disease progression remains unclear, oxidative stress is recognized as the most convincing mediator of second hit in NASH (4-6). Significantly elevated hepatic 8-oxodG in NASH compared with simple steatosis supports the hypothesis that oxidative stress may contribute to the pathogenesis of NASH. Because the hepatocytic 8-oxodG counts were significantly correlated with platelet count, oxidative stress may be related to disease progression in NASH, especially fibrogenesis. Seki et al. (4) also reported that hepatic oxidative stress formation as assessed by the level of 4-hydroxy-2′-2 nonenal was significantly increased with the progression of histologic fibrosis staging in NASH. The degree of hepatic fat deposit seems to be relevant to hepatic oxidative stress formation in NASH because hepatic 8-oxodG levels were positively correlated to the extent of steatosis in NASH. But steatosis alone could not cause the hepatic oxidative stress because the degree of hepatic steatosis was not significantly different between the NASH and simple steatosis, and steatosis and 8-oxodG levels were not correlated in simple steatosis patients. These results clearly indicate that second hit is necessary for the development from simple steatosis to NASH.

Some authors believe that iron may be the substrate of oxidative stress and could be responsible for the second hit in patients with NASH (23, 24). In steatotic livers, the saturation of β-oxidation by excess free fatty acids will ultimately lead to the generation of hydrogen peroxide, which in turn can be converted to highly reactive hydroxyl radicals in the presence of free iron via Fenton reaction (10). Indeed, there is strong evidence, from *in vitro* and *in vivo* studies, that iron overload enhances oxidative stress (25-27). Consistent with several previous findings (7-9), the present data showing that serum iron, transferrin saturation, and ferritin levels and the grade of hepatic iron staining (TIS) are significantly higher in NASH compared with simple steatosis also suggest that iron overload may be responsible for the second hit and pathogenesis of NASH. Quantitative analysis revealed that hepatocytic 8-oxodG levels were significantly correlated with these iron-related markers in NASH, strongly indicating that the increase in the body stored iron is specifically related to increased hepatocytic oxidatively generated damage to DNA in NASH patients.

Because serum insulin and HOMA-IR were significantly higher in NASH than in simple steatosis, and fasting glucose levels and HOMA-IR were significantly correlated with hepatic oxidative damage to DNA in NASH patients, another important factor for hepatic oxidative stress formation in NASH may be insulin resistance, as same as the iron overload. A strong association between iron overload and insulin resistance has been proposed. In fact, Mendler et al. (28) defined a syndrome of "insulin resistance-associated iron overload" in the presence of unexplained hepatic iron overload and at least one component of the insulin resistance. Insulin resistance also seemed to be closely linked to total body iron stores in the general population. Body iron stores are positively associated with the development of glucose intolerance and type 2 diabetes (29, 30). Iron overload and insulin resistance relationship also confirms the fact that iron depletion can improve insulin sensitivity (31-33). Iron overload can interfere with insulin signaling through the induction of reactive oxygen species, the latter impairing insulin uptake through a direct effect on insulin receptor function, by inhibiting the translocation of glucose

Table 5. Profile, phlebotomy, and changes in individual data after iron reduction therapy in patients with NASH

Patient no.	Age/ gender	Phlebotomy period (mo)/volume (mL)	BMI (kg/m^2)		ALT (IU/L)		Hemoglobin (g/dL)		Serum iron (μg/dL)		Ferritin (ng/mL)		TIS		8-oxodG ($/10^5$ μm^2)	
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	47/M	9/2,200	31.0	29.7	59	32	15.6	14.7	220	172	718	77	8	4	40.0	21.7
2	66/M	9/1,000	26.2	25.9	171	110	14.5	14.2	188	155	431	382	6	8	32.0	39.0
3	67/M	12/1,800	27.3	26.5	98	56	14.2	13.6	141	115	539	123	5	4	27.0	22.3
4	41/F	14/2,400	29.0	27.8	136	97	14.5	13.2	170	122	481	53	5	2	25.3	12.3
5	59/F	13/1,400	35.0	32.2	46	38	14.9	13.6	138	101	223	75	5	1	20.0	12.7
6	41/M	12/2,800	25.1	25.3	122	89	15.3	13.9	92	72	374	68	4	4	19.0	5.7
7	59/F	10/800	23.5	22.2	133	72	12.2	11.5	202	154	847	272	7	5	17.6	6.7
8	54/F	8/1,200	25.2	25.3	82	49	15.7	15.2	124	107	300	109	2	2	13.3	12.0
9	42/M	12/1,800	28.1	28.3	94	42	16.4	14.7	120	77	537	39	5	2	12.3	5.0
10	39/M	9/2,000	28.1	24.5	118	77	16.2	14.3	134	100	306	34	2	0	11.7	3.7
11	64/M	11/1,200	30.4	30.1	37	49	15.0	14.2	96	94	339	46	4	2	10.0	10.7
Mean			28.1*	27.1*	99.6†	64.6†	15.0‡	13.9‡	148§	115§	463‖	116‖	4.8¶	3.1¶	20.7**	13.8**

*Statistically significant difference at $P = 0.0222$ (paired t test).
†Statistically significant difference at $P = 0.0003$ (paired t test).
‡Statistically significant difference at $P < 0.0001$ (paired t test).
§Statistically significant difference at $P < 0.0001$ (paired t test).
‖Statistically significant difference at $P < 0.0001$ (paired t test).
¶Statistically significant difference at $P = 0.0113$ (paired t test).
**Statistically significant difference at $P = 0.0092$ (paired t test).

transporter GLUT4 to the plasma membrane (34, 35). The relation of insulin resistance and iron overload is also important in reverse, as insulin stimulates cellular iron uptake through increased transferrin receptor externalization (36, 37). It is also known that the glycation of transferrin decreases its ability to bind ferrous iron (38) and, by increasing the pool of free iron, stimulates ferritin synthesis. Glycated holotransferrin is additionally known to facilitate the production of free oxygen radicals, which further amplify the oxidative effects of iron (38). Reciprocally, the oxidative stress also induces both insulin resistance [by decreasing internalization of insulin (34)] and increased ferritin synthesis. Therefore, iron overload, insulin resistance, and oxidative stress may amplify each other and may compose the vicious cycle to progress liver injury in NASH.

The above-mentioned results prompted us to investigate the possibility of iron reduction for improvement of hepatic oxidative damage to DNA in NASH. Iron reduction (phlebotomy plus iron–restricted diet) therapy for NASH significantly reduced the serum ALT and hepatic 8-oxodG levels, suggesting the possibility of iron reduction for treatment option for NASH. Recently, Valenti et al. (33) reported that iron reduction also improved insulin resistance in 64 phlebotomized nonalcoholic fatty liver disease patients with hyperferritinemia. A randomized study also suggests that iron reduction may recover insulin action in type 2 diabetic patients (39). But in our treated NASH patients, iron reduction did not significantly affect insulin resistance state. Large randomized controlled studies, considering histology as final outcomes, are nonetheless required to determine the clinical effect of iron reduction therapy in patients with NASH before this therapy can be proposed.

In conclusion, iron overload, insulin resistance, and hepatic oxidatively generated damage to DNA tightly correlate each other in NASH patients, suggesting that these three factors may play an important role in the pathogenesis of NASH. Simple and inexpensive therapies, such as phlebotomy and iron–restricted diet, may be emerging as effective treatment options, which may lead to reduction of hepatocellular carcinoma incidence in NASH patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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内科外来における治療と指導法

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内科外来におけるアルコール医療は、アルコール性臓器障害を治療する役割を持つが、通常の内科診療とは異なる特徴がいくつかある。一つは飲酒期間が10年、20年といった長期にならないと症状が出ないためすでに依存が生じており、今までと同じ量を飲酒しているにもかかわらず突然症状が出たことに対し、患者が飲酒を原因と素直に受け止められない点である。二つ目は、個人差が大きく、他人は同じ飲酒量で臓器障害がないのに自分だけに発症したことに納得がいかない点。三つ目は、初期のうちは禁酒により速やかに改善するため、患者が病状を深刻に考えず、症状が消失すると通院を中断し、再飲酒に至る例が多い点である。

このような特徴から、アルコール性臓器障害の治療においては他の治療にはない困難性があり、投薬など治療内容は同じでも異なるアプローチが必要となる。

内科診療における治療の困難性

内科におけるアルコール医療の困難性は、患者に病識がなく、健康診断などで異常を指摘されても病気を治そうとする意思がなく、受診する段階ではかなり病状が進んでいる例が多いことと、障害が多臓器にわたる（表）ことに起因する。肝硬変や慢性膵炎、心筋症や脳神経障害など病状が進んだ段階ではアルコール依存症の可能性が高く、断酒が必要である。初診時から家族が同伴して禁酒を要望する場合も、依存症と考えてよい場合が多い。

比較的病状が進んでいない状態では、節酒できる可能性のある人もいるので、まず節酒を勧める。2週間ごとの通院で肝機能の推移を確認する。この際、家族を同伴させると効果的なことが多い。プレアルコーリック（家庭不和、無断欠勤、飲酒運転などのアルコール関連の社会的問題を持つが、連続飲酒発作や離脱症状のないもの）の段階で早期介入すれば依存症の予防につながり、また治療期間も短縮できる。内科外来で問題となるのは自ら節酒できない人

表　習慣性の大量飲酒に伴う臓器障害

消化器疾患	
食道	食道潰瘍、食道炎、食道がん、食道静脈瘤、Mallory-Weiss 症候群
胃・十二指腸	胃・十二指腸潰瘍、胃・十二指腸炎、急性胃粘膜病変（出血性胃炎）
小腸・大腸	びらん、下痢、吸収障害、大腸がん
肝臓	脂肪肝、肝炎（重症型アルコール性肝炎）、肝線維症、肝硬変、肝細胞がん
膵臓	急性膵炎、慢性膵炎
脳神経障害	
ビタミン欠乏症、Wernicke-Korsakoff 症候群、小脳変性症、ペラグラ、多発神経炎（下肢遠位部から侵され、鈍い持続痛）、アルコール性痴呆、アルコール性大脳萎縮（前頭葉に顕著）	
アルコール性筋症（ミオパチー）	
赤色筋、下肢近位筋に多い	
骨疾患	
骨粗鬆症、大腿骨骨頭壊死	
循環器疾患	
高血圧症、アルコール性心筋症、虚血性心疾患、不整脈	
造血器障害	
巨赤芽球性貧血、溶血性貧血、血小板減少	
代謝障害	
高中性脂肪血症、VLDL（超低比重リポ蛋白）上昇、pre-β リポ蛋白分画増加、高乳酸血症、高尿酸血症	

であるが、断酒の必要性が高い依存症患者ほど断酒が容易ではないという困難がある。節酒できない旨の自己申告があった場合や、肝機能の改善がない場合には依存症の可能性が高いことを説明し、精神科やアルコール依存症治療専門機関への紹介が必要となる。

アルコール依存症では多臓器に障害があることが多く、複数の医師で診察に当たることがある。この場合、専門医は多臓器にわたる病態を持つ患者に興味を持ちにくく、最終責任を誰が持つかが不明瞭となり、介入の遅れから依存症が進行するリスクが高い。

アルコール依存症の根底にある"否認"

問診で問題となるのが"否認"である。「自分の飲酒量、飲酒行動には全く問題ない」とする"完全否認"もあれば、「過剰飲酒は認めるが、依存症ではない」といった"縮小化する否認"もある。一般診療では患者が症状を過少申告することはないが、アルコール依存症では依存症と診断されることを嫌い、飲酒量は少なめに答え、臓器障害の病名を付けられることに安心し、症状を抑える投薬のみを希望することが多い。多くの内科医は、こうした患者を診ると診療に値しない患者と判断し、除外してしまう。

しかし、この"否認"こそがアルコール依存症特有のサインであり、その根底にあるものである。お酒をやめることはしらふで現実と直面することになり、飲酒はそれを回避するための心の防衛反応とも言える。仮に過剰飲酒を認めたとしても、夫の非協力性や、妻の理解のなさ、さらには会社の不合理などを理由に挙げ、自分の非は少ないことを強調する。患者が病気（依存症）であることを認め、治療（断酒）を受け入れることで初めて回復に向かう。ただし、回復した時点で、私は依存症でなかったのではないかという第二の否認に至る例も多いので安心してはいけない。

この否認の連鎖を断ち切ることが、治療につながることを理解しなければならない。家族の否認（「私のせいで夫は飲み過ぎてしまう」「私が何とかできる」など）も本人の否認を増強する。治療者（内科医）の否認（「肥満が半分くらい関係している」「食べ過ぎが一部この病気を悪化させている」「1合程度なら飲んでも治せる」など）も本人の否認を増強する。

内科外来での接し方

アルコール依存症の問診（特に飲酒歴の聴取）は診断のためだけでなく、患者に自分の飲酒習慣を振り返って反省してもらうための治療行為としても重要であり、機械的ではなく時間をかけて具体的に聞く必要がある。「1日にどのくらい飲みますか」ではなく、「最大でどれくらい飲みますか」「何を飲みますか」「ボトルは何日で空きますか」などと具体的な質問をする。「今はやめています」「最近は1合に減らしています」といった答えには、「いつからですか」と具体的な期間を聞く。それでも患者が過少申告することは多く、家族からの情報も重要である。忙しい外来診療の合間では困難を極めるため、看護師やソーシャルワーカーなどの協力を得る必要がある。

初診の段階で依存症と診断がつく患者には、「あなたの病気はアルコールが原因です。お酒をやめる決意があるなら一生懸命治しますが、もし、やめる気がないなら協力できません」ときっぱり言う。依存症患者には節酒指導は行わない。自信がなくても「やめます」と本人に言わせることが重要である。

2週間の禁酒から節酒指導へ

プレアルコーリックの段階でも、とりあえず2週間は禁酒してもらう。2週間禁酒できた場合は大いに褒め、食事がおいしい、体調が良いなど、禁酒して良かった点を聞き出す。

その後、節酒に移行した場合、飲んだ時間、量、誰と飲んだか、その時の気分など飲酒日記を毎日つけさせると良い。飲酒量が増えた時はどんな時か、反省の材料にも使える。二次会を断る、濃い酒は薄めて飲む、食事と一緒に飲む、行きつけの店の前を通らないで帰るなど、ただ「控えなさい」と言うだけでなく過剰飲酒を防ぐ方法を、日記をもとに具体的に検討する。

あまりに厳しい指導は外来通院の中断につながる可能性も高いので、まずは実行可能な目標を立てる必要がある。患者を叱るのではなく患者の自己決定を尊重し、節酒のために自分でできることを提案してもらう。少しずつでも改善しているなら肝機能などデータの推移をフィードバックする。うまくいっていないなら行動目標の切り替えを相談

する。本を読む、運動するなど、飲酒以外の生活習慣にも目を向けさせると良い。寝酒として飲酒習慣のある人には、抗不安薬を就寝前に処方してもよい。

多くの合併症（高脂血症、糖尿病など）は2週間の禁酒で軽快傾向を示すことが多い。安易な合併症治療の投薬は「自分は臓器障害なんだ」と飲酒問題から目をそらさせるリスクもあり、必要最低限に抑えるべきである。

節酒できない場合

節酒できない場合や2～3日で離脱症状が出たり、2週間の禁酒ができない場合は、依存症のことが多い。依存症と診断し、断酒の必要性を説明する。糖尿病などが合併していると、まずは食事指導から、などと逃げる人が多いので、「あなたの病気はアルコールが原因である」ときっぱり伝え、もう一生分の飲酒をしてしまったこと、酒なしでも楽しい生活が送れること、アルコール依存症は人生の脱落者ではなく、むしろうつ病と同じように働き過ぎ、ワークホリックの人がストレス解消の過程として移行することが多いこと、早期にきちんと治療すれば生命予後は良好なことを話す。肝硬変に至っても、飲酒を続ければ5年生存率は30%程度なのに対し、断酒すれば9割近い生存率があり、健常者とほとんど差がない。

プレアルコーリックなら初めは内科で経過を見てもよいが、依存症の場合は「あなたは依存症なのでアルコールの専門病院の受診が必要である」との旨をしっかりと説明する。腰痛に例えれば、初めは内科で痛み止めを出して様子を見ることもできるが、骨折なら直ちに整形外科を受診してもらうのと同様である。

精神科に偏見がある人が多いため、「入院する、しないは自分の希望でよいので、まずは専門医の意見を聞いてみては」と敷居を低くして専門医の外来を受診させる。先述の"否認"を打破する最も有効な手立ては、専門医の受診であることを認識しておく必要がある。

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METABOLISM, CANCER AND GENETICS

SPINK1, *ADH2*, and *ALDH2* gene variants and alcoholic chronic pancreatitis in Japan

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Key words
alcoholic pancreatitis, mutational analysis, pancreatic secretory trypsin inhibitor, pancreatitis, serine protease inhibitor Kazal type 1.

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Abstract

The serine protease inhibitor Kazal type 1 (SPINK1) is a potent antiprotease and an important inactivation factor of intrapancreatic trypsin activity. Loss of function by the *SPINK1* mutations leads to decreased inhibitory capacity. The significance of *SPINK1* mutations in alcoholic chronic pancreatitis (CP) in Japan and its functional role remain unclear. The aim of the present study was to clarify the incidence of *SPINK1*, alcohol dehydrogenase 2 (*ADH2*) and aldehyde dehydrogenase 2 (*ALDH2*) variants in CP patients in Japan. One hundred and 86 patients with CP, and 527 healthy volunteers were enrolled. Mutational analyses were performed by polymerase chain reaction–restriction fragment length polymorphism and direct sequencing. Serum pancreatic secretory trypsin inhibitor (PSTI) level was measured by radioimmunoassay. The frequencies of N34S and IVS3 + 2T > C in the *SPINK1* gene were significantly higher in patients with non-alcoholic CP (12.9% and 8.6%, respectively) than in normal subjects (0.37% and 0%). In total, 18 of 93 (19.4%) patients with non-alcoholic CP had at least one *SPINK1* mutation. Concerning alcoholic CP, we found IVS3 + 2T > C in a small number of patients (3.9%). Serum PSTI concentration was decreased in patients with the IVS3 + 2T > C mutation. The frequency of the *ADH2*2* allele in the alcoholic CP group was significantly higher than that in alcoholics without pancreatitis. The frequency of the *ALDH2*2* allele was significantly low in patients with alcoholic CP compared with healthy controls. In conclusion, *SPINK1* mutations were associated with non-alcoholic CP. Furthermore, we revealed the amount of wild-type PSTI was decreased in patients with IVS3 + 2T > C mutation. Variants of alcohol-metabolizing enzymes appeared in the relation to alcoholic CP.

Introduction

Chronic pancreatitis (CP) is a progressive inflammatory disease that eventually leads to impairment of exocrine and endocrine functions of the pancreas.[1,2] Long-term consumption of large amounts of alcohol is the main cause of CP. In Japan, more than half of CP is caused by alcohol, followed by 'idiopathic' which accounts for approximately 20% of the cases. However, all heavy drinkers do not always develop chronic pancreatitis, being < 1% in Japan and 5% in the USA.

The serine protease inhibitor Kazal type 1 SPINK1 (OMIM 167790, also called pancreatic secretory trypsin inhibitor [PSTI]) is a potent antiprotease that is thought to be an inactivation factor of intrapancreatic trypsin activity. In 2000, Witt *et al.* reported a transition mutation resulting in a substitution of asparagine by serine at codon 34 (N34S) in 18 of 96 (19%) unrelated German children and adolescents with CP.[3] The high frequency of N34S in CP patients has been confirmed by several studies.[4–12] But, the significance of *SPINK1* gene mutations in alcoholic CP remains unclear in Japan. PSTI has also been detected in some extrapancreatic normal tissues and in various cancers.[13] Since radioimmunoassays for pancreatic enzymes have been established, measurement of immunoreactive PSTI could be helpful both in diagnosis and assessment of acute pancreatitis and malignant diseases.[14,15] However, the correlation between serum PSTI level and *SPINK1* mutations has not been clarified.

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase 2 (ALDH2) play central roles in the initial stages of alcohol metabolism. The ADH are the homodimeric and heterodimeric isozymes whose subunits are encoded by the *ADH1*, *ADH2*, and *ADH3* genes. Polymorphisms with physiological significance exist in the *ADH2* and *ADH3* gene loci to different degrees in different ethnic groups. The *ADH2*2* allele, which encodes the more active β2-ADH subunit, predominates in the Asian population. This variant can produce acetaldehyde much faster than the usual ADH, encoded by the *ADH2*1* allele.[16] In the *ALDH2* gene, glutamine-lysine amino acid replacement caused by one nucleotide transition in exon 12 results in loss of catalytic activities. As is well known, the variant form of ALDH2 is found almost exclusively in populations of Asian origin, and approximately 45% of Japanese possess this type of ALDH2.[17–19]

In the present study, we aimed to clarify the incidence of *SPINK1*, *ADH2*, and *ALDH2* variants in alcoholic CP patients in Japan. In addition to studying the functional effect of *SPINK1*

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mutations, serum PSTI level in subjects with *SPINK1* mutations was examined.

Methods

Subjects

All enrolled subjects gave their informed consent according to the ethical guidelines of the Declaration of Helsinki. This study was approved by the Ethics Committee of Tohoku University School of Medicine. One hundred and 86 (149 males and 37 females) unrelated patients with CP were enrolled in this study. The diagnosis of CP was based on the standard diagnostic criteria for CP, consisting of imaging tests, exocrine function tests, and histological examinations. The diagnosis of hereditary pancreatitis was made on the basis of three or more relatives in two or more generations with CP and/or the confirmation of the *PRSS1* gene mutation. Patients with CP, in whom the criteria for hereditary pancreatitis were not met but there were at least two affected family members, was classified as familial pancreatitis. Idiopathic CP includes patients in whom no predisposing factor could be identified. Patients with alcohol consumption of more than 80 g/day for at least 2 years were classified as alcoholic CP. The etiologies of CP in this study were as follows: alcoholic ($n = 93$), hereditary ($n = 9$ from nine families), familial ($n = 12$ from 12 families), idiopathic ($n = 58$), autoimmune ($n = 13$), and divisum ($n = 1$). Among the patients with idiopathic CP, 21 patients who had developed pancreatitis before the age of 30 years were classified as early onset, whereas 37 patients who had developed pancreatitis after 30 years of age were classified as late onset. Five hundred and 27 unrelated healthy volunteers served as controls. All of the patients and controls were Japanese. The data on clinical course of pancreatic disease were based on physician's history and review of physical examination records.

Mutational analysis

Genomic DNA was extracted from peripheral blood leukocytes according to the standard protocol. Mutational analysis of *SPINK1*, *ADH2* and *ALDH2* genes was carried out by directional sequencing and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis, as we previously reported.[8,11]

Radioimmunoassay of serum PSTI

Blood samples were obtained from cases of chronic calcifying pancreatitis ($n = 3$), recurrent acute pancreatitis ($n = 1$), N34S mutation carrier ($n = 5$), and a healthy control ($n = 1$), after informed consent had been given. Blood samples for determination of PSTI were immediately separated and stored at −80°C until assayed. Serum PSTI was measured using a commercial radioimmunoassay kit (Ab Bead PSTI; Eiken Chemical Co., Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using the two-sided Fisher's exact test. A P-value < 0.05 was considered significant.

Results

SPINK1 mutations in Japan

In patients with pancreatitis, we found two types of mutation, [N34S; IVS1-37T > C] and [-215G > A; IVS3 + 2T > C], and two polymorphisms, -253T > C and 272C > T. The N34S mutation was found in 12 of 186 patients with CP (Table 1). Twelve CP patients carrying this mutation included five familial pancreatitis (one homozygous), six idiopathic CP (four early onset and two late onset), and one autoimmune pancreatitis. The heterozygous N34S mutation was found in two of 527 healthy controls. The prevalence of N34S was significantly higher in patients with familial pancreatitis (41.7%) and with idiopathic CP (10.3%), compared with healthy controls (0.37%).

The IVS3 + 2T > C mutation was found in 11 of 186 patients with CP (Table 1). Eleven CP patients carrying this mutation included one familial pancreatitis, seven idiopathic CP (two

Table 1 Frequencies of the N34S and IVS3 + 2T > C mutations in Japanese CP patients

Etiology	*n*	N34S						IVS3 + 2T > C					
		Total	ht	hm	Frequency*	Allele frequency	*P*-value	Total	ht	hm	Frequency*	Allele frequency	*P*-value
Alcoholic	93	0	0	0	0%	0%	–	3	2	1	3.2%	2.2%	0.0213
Non-alcoholic	93	12	10	2	12.9%	7.5%	< 0.0001	8	6	2	8.6%	5.4%	< 0.0001
Hereditary	9	0	0	0	0%	0%	–	0	0	0	0%	0%	–
Familial	12	5	4	1	41.7%	25.0%	< 0.0001	1	1	0	8.3%	4.2%	0.0476
Idiopathic	58	6	5	1	10.3%	6.0%	< 0.0001	7	5	2	12.1%	7.8%	< 0.0001
Autoimmune	13	1	1	0	7.7%	3.8%	0.0706	0	0	0	0%	0%	–
Divisum	1	0	0	0	0%	0%	–	0	0	0	0%	0%	–
Control†	527	2	2	0	0.37%	0.19%	–	0	0	0	0%	0%	–

CP, chronic pancreatitis; ht, heterozygous; hm, homozygous

*Frequencies of the N34S and IVS3 + 2T > C mutations in CP patients are shown as per patients.

†IVS3 + 2T > C mutation was examined in 240 healthy volunteers.

Table 2 Serum PSTI level and *SPINK1* mutations

Case	Age (years)	Sex	Diagnosis	*SPINK1* mutation	Serum PSTI (ng/mL)
1	32	F	CCP	N34S (hm)	6.0
2	33	F	CCP	N34S (ht)	8.8
3	76	M	Carrier	N34S (ht)	10.0
4	75	F	Carrier	N34S (ht)	9.5
5	48	M	Carrier	N34S (ht)	8.4
6	27	F	Carrier	N34S (ht)	7.0
7	25	M	Carrier	N34S (ht)	6.0
8	38	M	Recurrent AP	N34S (ht)/IVS3 + 2T > C (ht)	3.5
9	54	F	CCP	IVS3 + 2T > C (ht)	2.8
10	51	M	Healthy	None	8.1
Normal range					4.6–20.0

AP, acute pancreatitis; CCP, chronic calcifying pancreatitis; PSTI, pancreatic secretory trypsin inhibitor.

Table 3 *ADH2* genotype and allele frequency distributions

	Genotype			Allele	
	*2*1/2*1* *n* (%)	*2*1/2*2* *n* (%)	*2*2/2*2* *n* (%)	*2*1*	*2*2*
Chronic alcoholic pancreatitis ($n = 78$)*	8 (10.2)	29 (37.2%)	41 (52.6%)	0.287	0.712a
Chronic alcoholic pancreatitis ($n = 54$)[19]	2 (3.7%)	21 (38.9%)	31 (57.4%)	0.231	0.769
Alcoholic controls without pancreatitis ($n = 244$)[17]	84 (34.4%)	86 (35.2%)	74 (30.3%)	0.520	0.480b
Healthy controls ($n = 461$)	33 (7.2%)	160 (34.7%)	268 (58.1%)	0.245	0.755

ADH, alcohol dehydrogenase.
*Present study.
a *vs* b: $P < 0.001$.

homozygous, one early onset, six late-onset), and three alcoholic CP (one homozygous). The IVS3 + 2T > C mutation was not found in 240 healthy controls. The prevalence of IVS3 + 2T > C mutation was significantly higher in patients with familial pancreatitis (8.3%), with idiopathic CP (12.1%), and with alcoholic CP (3.2%) than in healthy controls (0%). Two patients with idiopathic CP were compound heterozygote, carrying both N34S and IVS3 + 2T > C mutations. In total, 18 of 93 (19.4%) patients with non-alcoholic CP had at least one *SPINK1* mutation. This prevalence in patients with non-alcoholic CP was significantly higher compared with alcoholic CP (3.2% $P = 0.0004$).

Serum immunoreactive PSTI level and *SPINK1* mutations

Serum immunoreactive PSTI levels were determined in four patients with pancreatitis, five N34S carriers and one healthy control (Table 2). Serum PSTI concentration was decreased in two cases with IVS3 + 2T > C mutation (3.5 and 2.8 ng/mL, respectively). Two CP patients with N34S mutation (one homozygous), five N34S carriers, and one healthy control showed normal levels of serum PSTI (4.6–20.0 ng/mL).

ADH2/ALDH2 polymorphisms in patients with alcoholic CP

The *ADH2*1/2*1*, *ADH2*1/2*2*, *ADH2*2/2*2* were seen in 10.2%, 37.2%, and 52.6% of patients with alcoholic CP, respectively (Table 3). The frequency of the *ADH2*2* allele was significantly higher in patients with alcoholic CP (71.2%) compared with alcoholics (48.0%) ($P < 0.001$). Surgical procedures were reported in 23 of 41 (56.1%) patients with the *ADH2*2/2*2* genotype; this proportion is higher than in those with the *ADH2*1/2*1* or *ADH2*1/2*2* genotype (32%). We found no significant differences among the group of *ADH2* genotypes with regard to diabetes mellitus, peptic ulcer, pancreatic pseudocyst, and dilatation of the common bile duct. The *ALDH2*1/2*1*, *ALDH2*1/2*2*, and *ALDH2*2/2*2* were seen in 95.3%, 4.7%, and 0.0% of patients with alcoholic CP, respectively (Table 4). The frequency of the *ALDH2*2* allele was significantly lower in patients with alcoholic CP (2.3%) compared with healthy controls (24.3%) ($P < 0.001$).

Discussion

SPINK1 is thought to be the first line of defense against prematurely activated trypsinogen, and the loss of SPINK1 function results in increased intrapancreatic trypsin activity.[3,4] We herein showed that the prevalence of the *SPINK1* mutations was high in patients with non-alcoholic CP, whereas the contribution was less evident in cases of alcoholic CP. This is in agreement with the results of previous studies in other Western countries showing limited contribution of the *SPINK1* mutation to the risk and severity of alcoholic CP.[5,20] Schneider *et al*. reported from the USA that the N34S mutation was detected in two of 32 (6.3%) patients with alcoholic CP.[20] In a European study, the N34S mutation was detected in 5.8% of the patients (16/274) with

Table 4 *ALDH2* genotype and allele frequency distributions

	Genotype of ALDH2			Allele	
	*2*1/2*1* *n* (%)	*2*1/2*2* *n* (%)	*2*2/2*2* *n* (%)	*2*1*	*2*2*
Chronic alcoholic pancreatitis ($n = 86$)*	82 (95.3%)	4 (4.7%)	0 (0.0%)	0.977	0.023 a
Chronic alcoholic pancreatitis ($n = 54$)[16]	46 (85.2%)	8 (14.8%)	0 (0.0%)	0.926	0.074
Alcoholic controls without pancreatitis ($n = 244$)[17]	210 (86.1%)	34 (13.9%)	0 (0.0%)	0.931	0.069
Healthy controls ($n = 461$)	268 (58.1%)	162 (35.1%)	31 (6.7%)	0.757	0.243 b

ALDH, aldehyde dehydrogenase.
*Present study.
a *vs* b: $P < 0.001$.

alcoholic pancreatitis and in 0.74% of the control population (4/540).[5] The relatively low frequency of *SPINK1* mutations in patients with alcoholic CP worldwide suggests that this genetic variant does not play a key role in the majority of patients. In the present study, the three patients with alcoholic CP carrying the IVS3 + 2T > C mutation had onset of pancreatitis at ages 28, 30, and 42, respectively. These ages of onset were earlier than the average age of 47 in our patients with alcoholic CP. Thus, *SPINK1* mutations may have an impact on the phenotypic presentation of alcoholic CP.

PSTI has also been detected in some extrapancreatic normal tissues and in various cancers.[13] The previous clinical applications revealed that serum PSTI was significantly elevated in acute pancreatitis, chronic relapsing pancreatitis, and in various malignant diseases.[14,15] In a further clinical study of serum PSTI determination, serum PSTI level remained within the normal range after total pancreatectomy.[21] Few patients with chronic pancreatitis or pancreatic cancer showed decreased concentrations,[15] although the pathophysiological role of the low PSTI level has not been fully clarified. In the present study, we found that serum PSTI levels were decreased in two cases with heterozygous IVS3 + 2T > C mutation (3.5 and 2.8 ng/mL, respectively).

We have previously examined the mRNA sequences of the *SPINK1* gene and revealed that the IVS3 + 2T > C mutation caused skipping of the whole of exon 3, leading to the loss of the trypsin-binding site.[10] Furthermore, the amount of wild-type transcript in a patient with heterozygous IVS3 + 2T > C mutation was found to be 62% of the healthy control.[12] The low wild-type PSTI level in patients with the IVS3 + 2T > C mutation confirms these previous researches showing that mRNA level was altered by this mutation.

Recently, the L14R mutation in the signal peptide of *SPINK1* was found in two families with hereditary pancreatitis.[22] Transfection experiments of the L14R mutation using human embryonic kidney cells indicated a complete loss of secretion. In addition, experimental demonstration revealed that the rare missense mutations, D50E, Y54H, and R67C reduce SPINK1 secretion by causing intracellular retention and degradation of SPINK1.[23] Serum wild-type PSTI content examined by specific radioimmunoassays might be decreased in patients with these rare missense mutations as well as the splicing variant.

The disease-relevant functional effect of the N34S mutation on SPINK1 structure and function has remained obscure. In the present study, serum PSTI level in patients with homozygous N34S or heterozygous N34S mutations was within the normal range. This finding confirms a previous study showing that the N34S mutation is not associated with alternative splicing[12] and does not alter SPINK1 folding/secretion.[23] A study comparing recombinant N34S and wild-type SPINK1 found no difference in trypsin activity or binding to trypsin.[24] Ironically, the functional defect caused by N34S, the most frequent *SPINK1* mutation, remains enigmatic awaiting further investigation.

As for the genotypes on alcohol-metabolizing enzymes, few reports have appeared on their relation to alcoholic pancreatitis.[17,18] In the present study, the frequency of the *ADH2*2* allele in the chronic alcoholic pancreatitis group (71.2%) was significantly higher than that in alcoholics without pancreatitis (48.0%). Our results agree with those reported by Maruyama *et al.*[18] and Matsumoto *et al.*[17] We also showed the *ADH2*2/2*2* genotype increases the frequency of pancreatic surgery. Matsumoto *et al.* reported that the frequency of the *ADH2*2* allele was high in cases with characteristic fibrosis of alcoholic pancreatitis.[17] However, a role of ADH2 in pancreatic cellular disorder is still not clear. The incidence of *ALDH2*1/*2* and *ALDH2*2/*2* was significantly reduced in patients with alcoholic CP (2.3%) compared with control subjects (24.3%). The inactive form of ALDH2 is considered to protect against alcoholism by allowing high levels of acetaldehyde to cause an adverse reaction.

In conclusion, we reported that *SPINK1* mutations were associated with non-alcoholic CP. The amount of wild-type PSTI was decreased in patients with IVS3 + 2T > C mutation. Further studies are necessary to clarify the disease-relevant functional effect of the N34S mutation on SPINK1 function.

Conflict of interest

No conflict of interest has been declared by the authors.

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膵炎は生活習慣病か?遺伝病か?

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要 旨

・症例対照研究の結果から,アルコールは急性膵炎と慢性膵炎の強い危険因子であり,喫煙も慢性膵炎発症と強い関連性が認められる。
・膵炎と関連した遺伝子異常として,カチオニックトリプシノーゲン遺伝子(*PRSS1*),アニオニックトリプシノーゲン遺伝子(*PRSS2*),キモトリプシンC遺伝子(*CRTC*),膵分泌性トリプシンインヒビター遺伝子(*SPINK1*),膵嚢胞線維症の原因遺伝子 *CFTR* やエタノール代謝に関連した遺伝子が知られる。
・アルコール性膵炎は生活習慣病としての一面を有するが,現在知られている膵炎と関連した遺伝子異常の役割は,特発性慢性膵炎以外は大きくはない。
・大量飲酒者のごく一部にしか膵炎が発症しないことから,いまだ明らかにされていない遺伝的素因が存在する可能性が考えられる。

膵炎は生活習慣病か?

厚生労働省の「発生要因に関する症例対照研究」によれば,急性膵炎や慢性膵炎の発症は飲酒量と有意な相関が認められる。非飲酒者に比べ,急性膵炎発症前24時間の飲酒量がエタノール換算で100 g以上のオッズ比は4.44であり,発症前1カ月の飲酒量が50〜99 g/日,100 g/日以上のオッズ比はそれぞれ3.50, 5.38と推定され,有意な量反応関係が認められている[1]。飲酒は慢性膵炎の強い危険因子でもあり,非飲酒者と比較し,現在飲酒者のオッズ比は,飲酒量が50〜99 g/日で6.72, 100 g/日以上では17.86にも達する[2]。

喫煙も慢性膵炎の強い危険因子である。非喫煙者に比べ,20本未満/日,20〜39本/日,40本/日以上の現在喫煙者のオッズ比はそれぞれ12.64, 5.48, 11.54であった。喫煙は飲酒と独立した慢性膵炎の危険因子と考えられている。

このようにライフスタイルと関連した後天的要因が膵炎発症や病態と密接に関連する結果が示される一方,大量飲酒者でも25年間に急性膵炎を発症するリスクは2〜3%,慢性膵炎においても発症頻度は大量飲酒者のたかだか5%以下と推定する報告もみられる。膵炎は生活習慣病としての側面を有する一方で,発症者には固有の遺伝的背景が存在するのではないかという疑問が生じる。

膵炎と関連した遺伝子異常とは?

近年明らかにされつつある膵炎と関連した遺伝子異常の多くは,腺房細胞内のトリプシンとその内因性の阻害因子,膵分泌性トリプシンインヒビター(PSTI)の不均衡をもたらし(図),細胞内トリプシン活性の持続が膵炎の本態である自己消化の閾値を下げるという分子機序を説明する。

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1．カチオニックトリプシノーゲン(CT)遺伝子(*PRSS1*)

遺伝性膵炎の原因遺伝子として，1996年に*PRSS1*エクソン3のミスセンス変異R122Hが初めて報告された。現在までに，世界中の200家系以上の遺伝性膵炎家系から約30種類の*PRSS1*の遺伝子変異が報告されているが，そのほとんどがアミノ酸置換を伴うミスセンス変異である。遺伝性膵炎全体の70～80%が*PRSS1*の遺伝子変異で説明され，R122Hが約80%弱，N29Iが約20%弱を占める。膵で生成されるヒトのトリプシノーゲンの2/3がCTである。遺伝子組み換えによって合成された変異CTの*in vitro*実験によって，変異の多くがCTの自己活性化を促進すること，R122Hなど一部の変異はCTの自己分解も阻害し，腺房細胞内でトリプシンの持続的活性化状態をもたらすと考えられている[3]。

CTの質的な異常に加え，最近*PRSS1*，*PRSS2*を含む広い領域の遺伝子重複，3重複が慢性膵炎の原因となる家系や症例が報告されている。Gene dose effectによって，腺房細胞内のトリプシノーゲンの過剰生成が膵炎の原因になる可能性が考えられる[4]。

2．アニオニックトリプシノーゲン(AT)遺伝子(*PRSS2*)

ヒトのトリプシノーゲンの1/3を占める主要な蛋白分解酵素の一つである。最近，*PRSS2*エクソン4の多型G191Rが，健常人に比べて慢性膵炎症例で有意に低いとする結果が報告された。G191Rによって，ATの分子内にトリプシンに対する感受性部位が新たに形成されるため自己分解が亢進し，生成されたトリプシンが活性を速やかに失うことが，慢性膵炎発症に保護的に働くとされる[5]。

3．キモトリプシンC遺伝子(*CTRC*)

トリプシノーゲンやトリプシンを分解する酵素として古くから想定されていたenzyme Yの本体がキモトリプシンCであることが明らかにされた。*CTRC*の多型R254WやK247-R254delの頻度が健常人(0.7%)に比べて慢性膵炎患者(3.3%)で有意に高く，アルコール性肝炎患者(0.7%)に比べても有意に高い(2.9%)ことが明らかにされた。機能解析から，これら多型はCTRCの機能障害や生成障害をもたらすと考えられる。CTRCの活性低下により，腺房細胞内で生成されたトリプシンはCTRCによる分解を免れ，細胞内活性を持続させることが膵炎を起こしやすくする[6]。

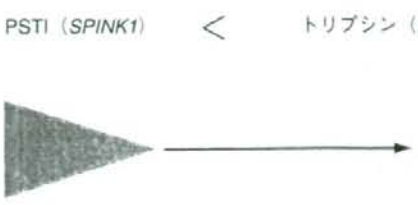

図　膵炎と関連した遺伝子異常の分子機序

4．膵分泌性トリプシンインヒビター(PSTI)遺伝子(*SPINK1*)

トリプシンの触媒部位と安定に結合し，その活性を抑制する内因性のプロテアーゼインヒビターである。2000年に若年発症の特発性膵炎や家族性膵炎患者に高頻度に遺伝子変異が認められることが明らかにされた。もっとも多い変異は，*SPINK1*エクソン3のN34Sであり，日本人の若年発症特発性慢性膵炎の23.8%，家族性膵炎の41.7%に認められる。イントロン3のIVS3+2T>C変異も日本人特発性慢性膵炎の11.4%に認められ，日本人に特徴的に多い変異である。

IVS3+2T>CはmRNAのスプライス供与部位の変異であり，mRNAの生成異常からPSTI活性をもたない異常蛋白が産生される[7]。L14R，L14PはシグナルペプチドのPSTIの変異をもたらし，PSTIの細胞内分解が促進される。D50E，Y54H，R67C，R65Qは，PSTIのアミノ酸鎖の折りたたみ異常から細胞内貯留と分解を起こす。このように，*SPINK1*変異はPSTIの機能や量の低下をもたらすが，もっとも多いN34Sがなぜ膵炎と関連するかはいまだに不明である。

5．その他の遺伝子

1）*CFTR*

膵嚢胞線維症(CF)の原因遺伝子*CFTR*には1,000種類もの遺伝子変異が認められるが，アミノ酸置換を伴うミスセンス変異やエクソン9の

表1　アルコール性，特発性膵炎における *SPINK1* 変異の頻度

			アルコール性	特発性	対　照
急性膵炎	*SPINK1*	N34S	2/43(4.7%)	3/64(4.7%)	1/165(0.6%)
		IVS3+2T>C	1/43(2.3%)	1/64(1.6%)	0/165(0%)
慢性膵炎	*SPINK1*	N34S	0/93(0%)	6/58(10.3%)**	2/527(0.37%)
		IVS3+2T>C	3/93(3.2%)*	7/58(12.1%)**	0/240(0%)

vs 対照　**p<0.01，*p<0.05

スプライス枝分かれ部位に位置する TG の繰り返し数とそれに続くチミジン数(TG)nTn が，CFTR の機能障害や mRNA のスプライス異常をもたらし，慢性膵炎発症と関連する可能性が示されている。しかし，白人に比べ日本人における *CFTR* の変異頻度は極めて低く，膵炎の病態における意義は不明である[8]。

2）*ADH*，*ALDH*

エタノールの酸化的代謝過程で生成されるアセトアルデヒドや活性酸素は細胞障害性に働く。この代謝をつかさどるアルコール脱水素酵素(ADH)やアルデヒド脱水素酵素(ALDH)は，膵腺房細胞にも存在する可能性が示されている。ADH や ALDH にはいくつかのクラスやアイソザイムが知られているが，特に *ADH2* と *ALDH2* はエタノールやアセトアルデヒドの代謝速度を規定するため，これらの遺伝子多型は腺房細胞の細胞障害をもたらし，膵炎の発症機序に関与すると考えられる。

アルコール性膵炎に遺伝子異常は関連するか？

1．アルコール性膵炎と *SPINK1*

SPINK1 遺伝子変異は健常人にも 1%以下の頻度で観察されるため，膵炎の比較的普遍的な遺伝的素因を形成する可能性がある。急性膵炎[9]と慢性膵炎[10]について *SPINK1* 遺伝子の N34S と IVS3+2T>C 変異を，アルコール性と特発性に分けて頻度を検討した(**表 1**)。

アルコール性急性膵炎における N34S，IVS3+2T>C の頻度はそれぞれ 4.7%，2.3%であり，健常対照群に比べて高い傾向は示したが有意差は得られなかった。また，特発性とアルコール性の頻度はほぼ同様であり，差は得られなかった。一方，特発性慢性膵炎における N34S，IVS3+2T>C の頻度は 10.3%，12.1%と対照群に比べて明らかに高く，膵炎発症と強い関連性を示した。アルコール性慢性膵炎では，IVS3+2T>C のみ膵炎と弱い関連性を示した。

以上の結果から，*SPINK1* 遺伝子変異は特発性慢性膵炎の強い危険因子の一つと考えられるが，アルコール性慢性膵炎の発症には飲酒自体の影響が強く現れる。また，急性膵炎では *SPINK1* 遺伝子変異が発症に一部関与する可能性は考えられるが，アルコール性と特発性で変異頻度に差が認められず，アルコールと *SPINK1* 変異の発症機序における役割は慢性膵炎とは異なる可能性が考えられた。

2．アルコール性膵炎と *ADH2*，*ALDH2*

アルコール性慢性膵炎における *ADH2* と *ALDH2* の遺伝子多型を膵炎のない大量飲酒者と比較した(**表 2**)[10]。アルコール性慢性膵炎患者における *ADH2*2* アレル頻度は 71%であり，膵炎を有しない大量飲酒者の 48%に比べて有意に高い頻度であった。また，アルコール性慢性膵炎患者における *ALDH2*2* のアレル頻度は 2.3%であり，膵炎のない大量飲酒者の 6.9%と差はなく，健常対照群の 24.3%に比べて有意に低い値を示した。

*ADH2*2* は *ADH2*1* に比べてエタノール酸化能が高いとされ，摂取されたエタノールは速やかにアセトアルデヒドに代謝される。*ALDH2*2* は非活性型酵素でアセトアルデヒドを代謝できず，飲酒後の顔面紅潮，心悸亢進，血圧低下，頭痛，悪心などフラッシング反応を生じることにより大量飲酒を抑制する。アルコール性慢性膵炎に多い *ADH2*2*，*ALDH2*1* は，大量飲酒者でエタノー

表2　アルコール性慢性膵炎における *ADH2*, *ALDH2* 多型の頻度

	ADH2 genotype			Allele frequency		
	2*1/2*1	2*1/2*2	2*2/2*2	2*1	2*2	
慢性アルコール性膵炎(n=78)	8(10.2%)	29(37.2%)	41(52.6%)	0.287	0.712	**
非膵炎アルコール摂取者(n=244)#	84(34.4%)	86(35.2%)	74(30.3%)	0.520	0.480	
健常対照(n=461)	33(7.2%)	160(34.7%)	268(58.1%)	0.245	0.755	
	ALDH2 genotype			Allele frequency		
	2*1/2*1	2*1/2*2	2*2/2*2	2*1	2*2	
慢性アルコール性膵炎(n=86)	82(95.3%)	4(4.7%)	0(0.0%)	0.977	0.023	**
非膵炎アルコール摂取者(n=244)#	210(86.1%)	34(13.9%)	0(0.0%)	0.931	0.069	
健常対照(n=461)	268(58.1%)	162(35.1%)	31(6.7%)	0.757	0.243	

#文献[11]参照　　**p<0.01

ルが速やかにアセトアルデヒドに変換されることによる膵障害を想定させる。

治療上の意義

アルコールは急性膵炎，慢性膵炎の後天的な強い発症要因である。最近明らかにされた *SPINK1* や *CTRC* の遺伝子変異は，アルコール性膵炎の弱い遺伝的背景となる可能性は考えられるが，膵炎患者に認められる頻度は低い。したがって，これらの遺伝子解析が，アルコール摂取者の中から膵炎高危険群を設定する検査法としての意義は低いと考えられる。*SPINK1* 遺伝子変異は，若年発症の特発性慢性膵炎や家族性膵炎で高い頻度を示し，むしろ若年で急性膵炎を繰り返すような原因不明の膵炎症例で遺伝的素因を明らかにし，禁酒・禁煙などライフスタイルの改善，指導による膵炎の進展防止と長期予後の改善を目指した治療に役立てるのが妥当と考えられる。

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アルコールと膵炎
――酒を飲みすぎると膵炎になるか?
Alcohol and pancreatitis

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◎アルコールは急性および慢性膵炎の主要な成因である．最近の全国調査では急性膵炎の37.3%，慢性膵炎の67.7%がアルコール性とされる．アルコールによる膵炎発症機序として蛋白塞栓説など，さまざまな仮説が提唱されているが，反論も少なくなくいまだ確立されていない．膵炎を発症するのは大酒家のうちたかだか数%にすぎないとされる．したがって，臨床的に膵炎が成立するためにはもともと何らかの先天的素因があり，アルコールはそれを増強する後天的要因のひとつと考えたほうが妥当である．先天的素因としてアルコールの酸化的代謝に関連するアルコール脱水素酵素や非酸化的代謝に関連するcarboxyl ester lipase，異所性に活性化されたトリプシンを阻害する膵分泌性トリプシンインヒビターなどの遺伝子多型が注目されている．

Key word：アルコール，膵炎，遺伝子多型，SPINK1，carboxyl ester lipase

1878年Friedreichは，膵腺房間の線維増生と腺組織の減少・消失を特徴とする膵の慢性炎症について記載し，これを“大酒家の膵(drunkard pancreas)”とよんでいる[1]．飲酒と膵炎の密接な関連が明らかにされたのは，1938年にWeinerら[2]が剖検で急性出血性膵炎の2/3に大量飲酒の既往を報告して以来である．また，Comfortら[3]は1946年に慢性膵炎の疾患概念を提唱したが，このなかで疾患背景として長期に及ぶ飲酒の存在を指摘している．このように膵炎とアルコールの関連は疾患概念の提唱とほぼ時を同じくして指摘されている．その後の疫学調査でも飲酒と膵炎の因果関係を強く示唆する報告は数多い[4]．各国における剖検例に占める慢性膵炎の頻度はその地域のアルコール消費量とよく相関することや，欧米ではアルコール消費量の増加に一致して膵炎の頻度が増加していること，膵炎発症のリスクの対数は飲酒量と直接的な相関を示すこと，剖検時に発見される膵炎の頻度はアルコール常用者では非飲酒者の50倍にも及ぶこと，飲酒期間が長いほど膵炎発症のリスクが高いこと，などが報告されている．

しかし，飲酒量や飲酒期間については個人差が大きく，また大酒家のうち膵炎を発症するのはたかだか数%にすぎないとされる．一方，急性膵炎や慢性膵炎患者のなかには機会飲酒程度の人やまったく酒を飲まない人も少なくない．すなわち，臨床的に膵炎が成立するためにはもともと何らかの先天的素因があり，アルコールはそれを増強する後天的要因のひとつと考えたほうが妥当である．

本稿ではアルコールによる膵傷害について，最近研究の進むアルコール膵炎を起こしやすい先天的素因＝遺伝的背景を含めて概説する．

アルコール性膵炎の実態

アルコールは急性および慢性膵炎の主要な成因である．厚生労働省難治性膵疾患に関する調査研究班が行った2003年の全国調査[5]では，2003年1年間における急性膵炎の受療患者は35,300人(95%信頼区間：30,500～40,000人)と推定され，調査票を回収しえた1,779例の成因はアルコール性が37.3%ともっとも多く，男女別では男性患者