for A9, AB045568 for A20, AB045671 for A68, AB045680 for A75, AB045681 for A77, AB045366 for A162, AB045572 for A303, AB045646 for A304, AB045648 for A306, AB045649 for A307, AB045678 for A712 and AB045679 for A713.

2B

AB047652 for A1, AB047671 for A5, AB047660 for A204, AB047661 for A205, AB047662 for A206, AB047669 for A414, AB047673 for A601, AB047654 for A159, AB047655 for A160, AB047656 for A161, AB047663 for A302, AB047680 for A811, AB047675 for A7, AB047681 for A9, AB047658 for A20, AB047674 for A68, AB047678 for A75, AB047679 for A77, AB047657 for A162, AB047664 for A303, AB047665 for A304, AB047666 for A306, AB047667 for A307, AB047676 for A712 and AB047677 for A713.

2C

AB082174 for A1, AB082130 for A5, AB0821323 for A204, AB082133 for A205, AB082134 for A206, A082135 for A414, AB082137 for A601, AB082139 for A159, AB082140 for A160, AB082141 for A161, AB082145 for A302, AB082147 for A811, AB082148 for A7, AB082149 for A9, AB082150 for A20, AB082154 for A68, AB082155 for A75, AB082156 for A77, AB082160 for A162, AB082165 for A303, AB082166 for A304, AB082167 for A306, AB082168 for A307, AB082171 for A712 and AB082172 for A713.

Phylogenetic analysis

To determine the heterogeneity of the viral sequences obtained from the 25 patients, a phylogenetic tree was constructed by the neighbour-joining method. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times. These analyses were conducted using a computer program, GENETYX-MAC version 10.1 (Software Development, Tokyo, Japan).

Statistical analysis

Differences in proportions among the groups were compared by Fisher's exact probability test, Student's *t*-test and Welch's *t*-test (DA STATS version 1.0, Nagata O, Tokyo, Japan).

Results

Clinicopathological characteristics of the patients

The characteristics of the 25 patients with hepatitis A analysed for HAV 5′NTR, 2B and 2C at admission are summarized in Table 1. None of the cases was associated with an epidemic.

Differences in the mean age, sex and presence of chronic liver disease among FH, AHs and AH, and between FH+AHs and AH, were not statistically significant. Serum was sampled 2–17 days after clinical onset. The mean ALT level was higher in AHs than that in AH ($P = 0.002$), and in FH+AHs than that in AH ($P = 0.003$). The mean prothrombin time was prolonged in FH compared with AHs ($P = 0.002$), FH compared with AH ($P < 0.001$), AHs compared with AH ($P < 0.001$) and FH+AHs compared with AH ($P < 0.001$). The mean total bilirubin level was higher in FH than that in AHs ($P = 0.049$).

Table 1. Characteristics of patients

	FH	AHs	AH
n	7	5	13
CLD	1†	1†	3†
Recovery/death	3/4‡	5/0‡	13/0‡
Sex (M/F)	3/4†	5/0†	7/6†
Age*	44.1 ± 13.5†	36.8 ± 12.9†	39.5 ± 9.1†
PT (%)*	16 ± 7§	34 ± 8§	63 ± 20§
ALT (IU/L)*	6337 ± 3838¶	6165 ± 1718¶	2873 ± 1733¶
T-Bil (mg/dl)*	9.4 ± 7.6‖	2.3 ± 0.8‖	5.0 ± 2.3‖

*Mean ± SD.

†Statistically not significant.

‡Statistically significant between FH and AH ($P = 0.007$) by Fisher's exact probability test.

§Statistically significant between FH and AHs ($P = 0.002$) by Student's *t*-test, FH and AH ($P < 0.001$) by Welch's *t*-test, and AHs and AH ($P < 0.001$) by Welch's *t*-test.

¶Statistically significant between AHs and AH ($P = 0.002$) by Student's *t*-test.

‖Statistically significant between AHs and FH ($P = 0.049$) and AHs and AH ($P = 0.002$) by Welch's *t*-test.

AH, acute hepatitis; AHs, severe acute hepatitis; ALT, alanine aminotransferase; CLD, chronic liver disease; FH, fulminant hepatitis; PT, prothrombin time; T-Bil, total bilirubin.

Four of seven patients with FH died of hepatic failure, and all patients with AHs and AH recovered ($P = 0.007$). All seven FH cases needed artificial liver support (plasma exchange and haemodiafiltration). Four (16%) patients – two (28%) with FH and two (15%) with AH – had acute renal failure and were treated by haemodiafiltration.

Two patients with AH were positive for HBsAg and antibody to HBe, and one patient with AH was positive for anti-nuclear antibody, but they showed a typical hepatitis A course. IgM anti-EBV, IgM anti-HSV, IgM anti-CMV, anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody-1 and anti-mitochondrial antibody were negative in all examined cases of FH and AHs. One FH patient and one AHs patient had histories of heavy alcohol consumption. One male patient with AH was homosexual.

Histological examination was performed in all seven FH cases, two of five AHs cases and seven of 13 AH cases in the convalescent phase or postmortem. In the FH cases, liver histology revealed massive necrosis in three patients and submassive necrosis in one. Liver histology in the two patients with histories of heavy alcohol consumption showed pericellular fibrosis, consistent with alcoholic liver disease. The histological findings of the other cases showed AH to be in a residual phase or subsiding.

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Phylogenetic analysis

The results of phylogenetic analysis are shown in Figures 1 and 2. Four FH (A204, A601, A414 and A1) and two AHs (A160 and A159) were located in the near parts of the phylogenetic trees (Fig. 2).

The clinical backgrounds, and the biochemical and viral characteristics are shown in Table 2. As described above, none of them were associated with an epidemic. Two of the FH patients died and the others recovered. HAV RNA was quantified by real-time RT-PCR in five of these six patients. Our other recent study of HAV RNA quantification revealed that the mean viral load in > 60 AH at admission was 2.75 ± 1.55 log copies/ml (20), and so these five patients had comparatively higher viral loads (4.35 ± 0.81 log copies/ml) ($P = 0.03$). The HAV genotype was IA in all patients, similar to the majority of Japanese patients in general.

Discussion

Although the severity of hepatitis A varies, it is not clear why it is more severe in some patients than that in others. It is thought that disease severity may be dependent on certain characteristics of the individual patients. It has been reported that ageing and underlying chronic liver disease could be factors that increase hepatitis A severity (21). Vento *et al.* (22) reported that patients with chronic hepatitis C had a substantial risk of FH and death associated with HAV superinfection.

During an urban epidemic in the US, it was described that hepatitis A caused serious illness and death and that complications were more frequent in patients 40 years of age and older, but that young healthy persons were also at risk for severe complications (23). A cluster of fulminant hepatitis A was reported, relating the severity of the infection in three siblings to the virulence of HAV, as the patients were all healthy before the infection and their illness followed a similar course (11).

In the past several years, increasing numbers of patients with sporadic hepatitis A, especially the more severe forms, have visited our hospital, but our analysis of factors possibly contributing to the severity of the disease failed to reveal any significant differences in patient characteristics including age (2, 3), suggesting that viral factors might determine the severity of the disease. To identify possible differences in hepatitis A viruses for different types of hepatitis, we analysed the HAV genome in sera from hepatitis A patients with various clinicopathological features. Our analysis of whole HAV genomes from three cases of FH and three cases of AH indicated possible associations between the severity of hepatitis A and the nucleotide substitutions in 5′NTR and the amino acid (aa) substitutions in 2B, although there were no unique nucleotide or aa substitutions. On the other hand, it was reported that mutations in 5′NTR, 2B and 2C of HAV were associated with cytopathic variants in cultured cells, and virulence in tamarins, as described above (9, 10). These various observations led us to analyse these three regions of HAV in greater numbers of clinical samples (14, 17, 18).

In our analysis of 5′NTR, FH and AHs patients had fewer nucleotide substitutions than AH in the central part of 5′NTR ($P < 0.001$) (14). Several regions of 5′NTR, including the pyrimidine-rich tract and internal ribosomal entry site, have been examined for possible correlations with replication of HAV RNA *in vitro*, and it has been reported that HAV strains adapted to cell culture systems have mutations in 5′NTR and the P2 region (8), and mutations in 5′NTR significantly enhanced growth of the virus in a cell culture system (24). Thus, nucleotide variations in 5′NTR may influence replication of the virus and thereby affect virulence.

In 2B, there seemed to be more mutations in the strains obtained from FH and AHs patients than in those obtained from AH patients in the central part (18). On the basis of cell culture studies, substitutions in the sequence of 2B protein have been suggested to be associated with the replication capability of the virus. One nucleotide substitution at nt 3889 in 2B, which changed Ala to Val in 2B-216, is responsible for differences in the growth rate of the virus along with the nucleotide substitutions in 2C and/or 5′NTR (25, 26). A substitution at the same nt 3889 appeared from the early stage of replication enhancement in cultured cells, and several HAV strains showed a cytopathic effect (8). An Ala-to-Val substitution in 2B-216 was not observed in our study.

In 2C, FH patients had fewer aa substitutions than AH patients ($P < 0.05$) (17). This indicates that viruses with fewer aa substitutions in 2C may be more virulent in comparison with strains with more aa substitutions. 2C is a multifunctional protein and is involved in replication of the viral genome. Analysis of the primary aa sequence of 2C shows homology with a family of proteins that contains a nucleoside triphosphate (NTP)-binding motif. This motif consists of elements 'A' and 'B'. The residues mutated within the conserved A and B sites of the NTP-binding motif are critical in RNA replication and virus proliferation (27). Elements A and B were conserved in all patients except one of FH. 2C is also suggested to be involved in the rearrangement of cellular membranes (28). The simian HAV 2C gene was reported to be required for virulence in tamarins (10). Thus, subtle substitutions in 2C might influence the replication capability of the virus and thereby affect virulence. We could not find specific nucleotide or aa substitutions in any of the regions.

In the present study, patients with FH had fewer nt substitutions in 5′NTR, and had a tendency to have more aa substitutions in 2B, and fewer aa substitutions in 2C, than patients with AH, and four FH and two AHs were located in the near parts of the phylogenetic trees, indicating the association between severity of hepatitis A and genomic variations in 5′NTR, 2B and 2C of HAV. In these patients, HAV load was higher than that of AH

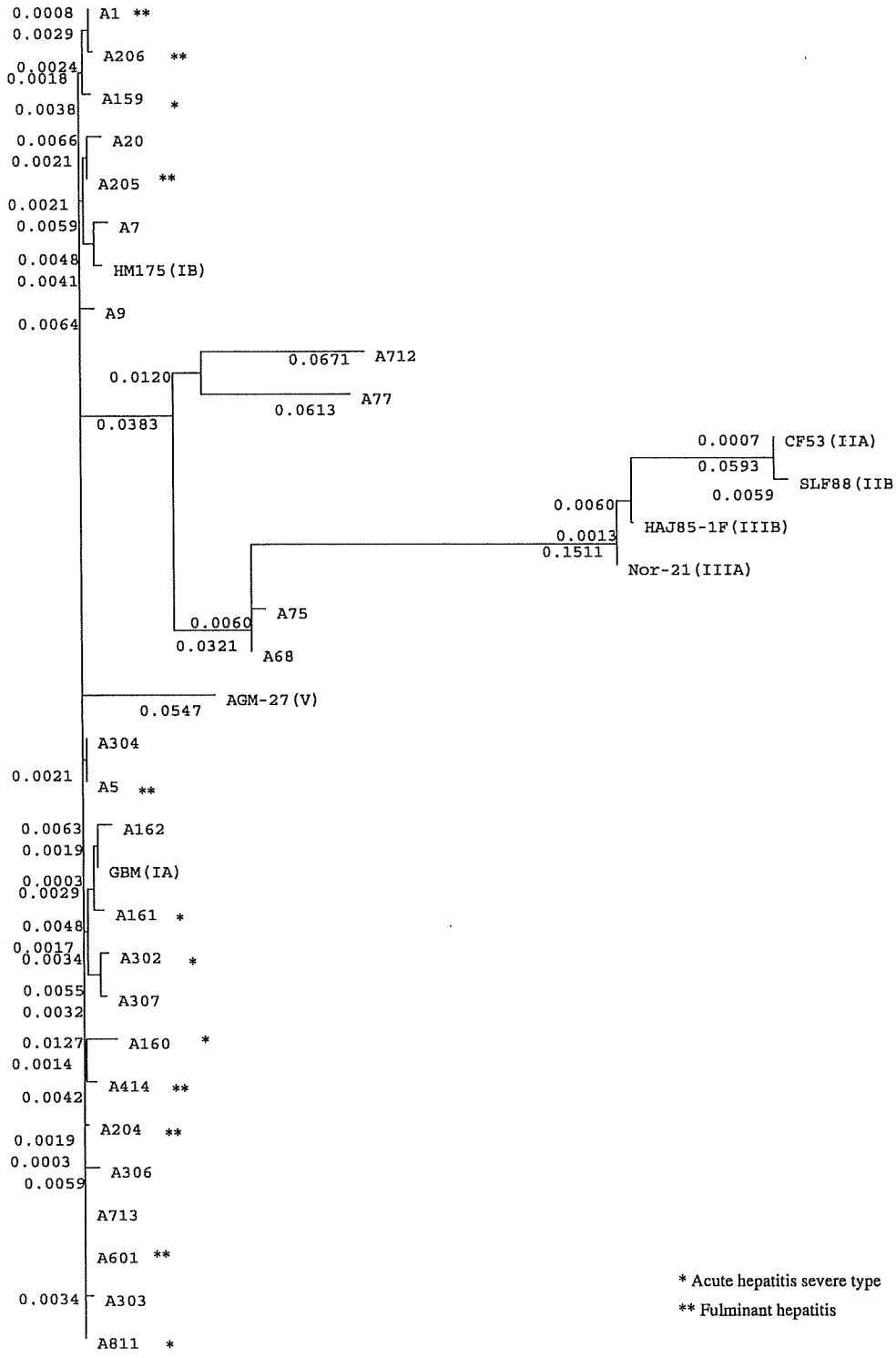

Fig. 1. Genetic relatedness between individual hepatitis A virus (HAV) strains between nucleotides 200 and 500 of the 5′ nontranslated region recovered from 25 patients and HAV reference strains GBM (subgenotype IA), HM175 (subgenotype IB), CF53 (subgenotype IIA), SLF88 (subgenotype IIB), Nor-21 (subgenotype IIIA), HAJ85-1F (subgenotype IIIB) and AGM27 (genotype V). Numbers beside the phylogenetic roots are the results of bootstrap analyses.

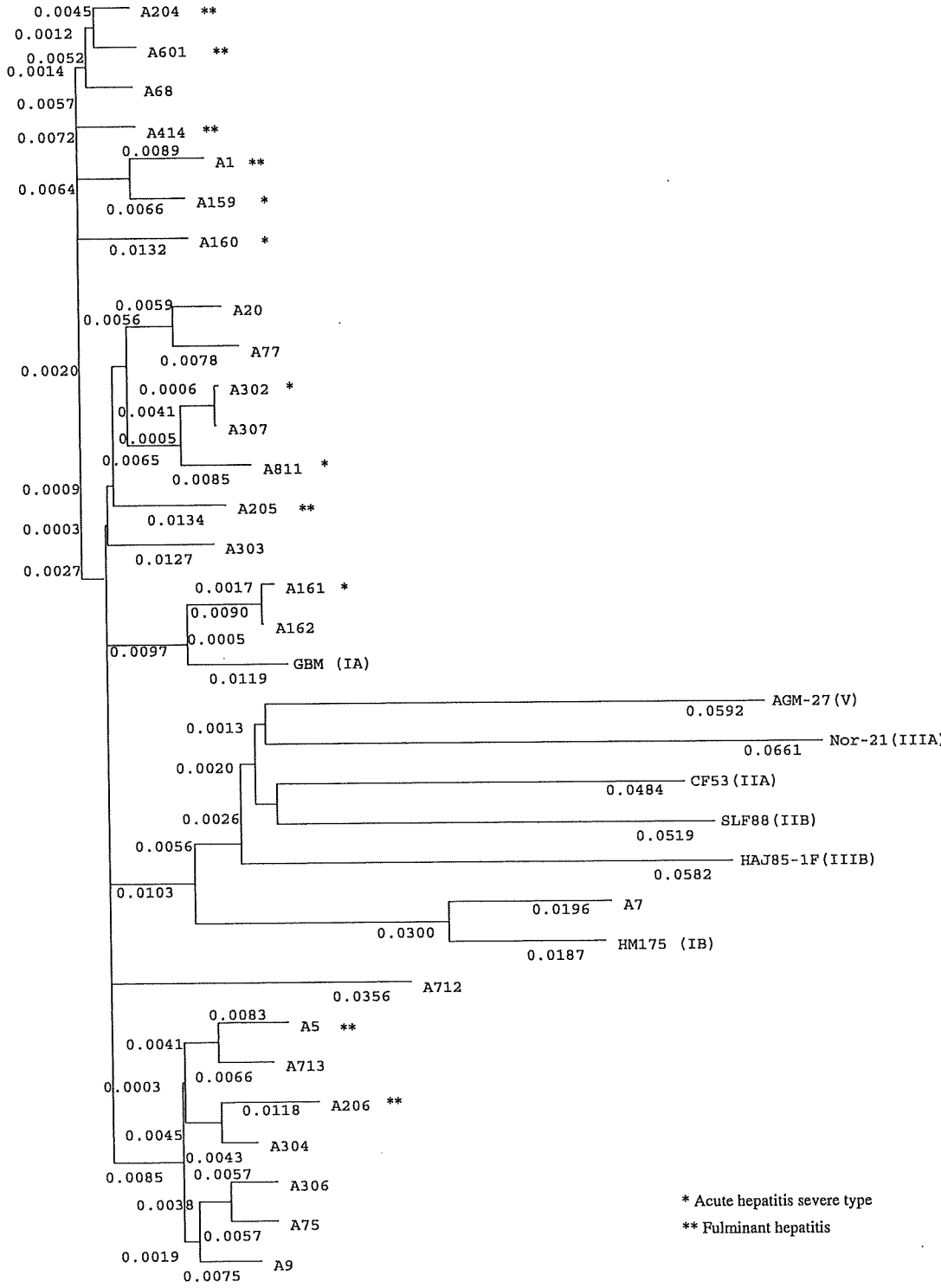

Fig. 2. Genetic relatedness between individual hepatitis A virus (HAV) of entire 2B and 2C recovered from 25 patients and HAV reference strains GBM (subgenotype IA), HM175 (subgenotype IB), CF53 (subgenotype IIA), SLF88 (subgenotype IIB), Nor-21 (subgenotype IIIA), HAJ85-1F (subgenotype IIIB) and AGM27 (genotype V). Numbers beside the phylogenetic roots are the results of bootstrap analyses.

Table 2. Clinical, biochemical and viral characteristics of six patients with fulminant and severe hepatitis located in the near parts of the phylogenetic tree

Patient	Diagnosis	Age/sex	Origin	Onset	Outcome	ALT (IU/L)	T-Bil (mg/dl)	PT (%)	IgM-HA (cut-off index)	Viral load (log copies/ml)	Days from onset
A204	FH	39/F	Tohoku	February 1990	Death	4470	5.3	10	3.8	ND	3
A601	FH	64/F	Shinetsu	January 1997	Death	12 500	7.0	13	2.9	3.7	9
A414	FH	49/M	Shinetsu	January 1989	Recovery	5276	26.3	13	+	5.0	7
A160	AHs	39/M	Kanto	June 1998	Recovery	9164	1.6	38	3.1	5.1	4
A1	FH	29/M	Kanto	March 1992	Recovery	1175	7.3	17	5.1	3.3	6
A159	AHs	50/M	Kanto	May 1998	Recovery	5655	2.5	20	4.6	4.6	5

Patient	Genotype	5′ NTR homology (%)	2B nt homology (%)	2C nt homology (%)
A204	IA	99.0	93.8	90.0
A601	IA	99.3	94.3	89.3
A414	IA	98.7	95.0	88.6
A160	IA	97.7	95.2	88.3
A1	IA	98.7	96.0	88.5
A159	IA	98.7	96.9	88.8

Homology, sequences were compared with wild-type HAV genotype IA strain GBM.
AH, acute hepatitis; AHs, acute hepatitis severe type; ALT, alanine aminotransferase; FH, fulminant hepatitis; 5′NTR, 5′-nontranslated region; ND, not done; nt, nucleotide; Ti-Bil, total bilirubin.

patients. Rezende *et al.* (29) reported that HAV-related liver failure is because of an excessive host response associated with a marked reduction in viral load, and there is a discrepancy between their data and ours. But they did not show the time points of serum sampling that represent critical data about viraemia in AH, and so we cannot discuss the discrepancy.

Thus, genetic variations not in one specific region but in 5′NTR, 2B and 2C might cooperatively influence replication of the virus and thereby affect virulence. Our findings are in accordance with the basic reports that the pathogenicity of HAV could be related to cooperative mutations within 5′NTR and P2 in cultured cells and simians, and the clinical finding that there has been only one report about a cluster of fulminant hepatitis A, unlike the many reports of clusters of fulminant hepatitis B.

Our current study suggests that both viral and host factors should be considered and examined when discussing the mechanisms responsible for the severity of hepatitis A. Further, we should examine several portions of the HAV genome including 5′NTR, 2B and 2C rather than focus on one specific region when analysing viral factors. Our study also suggests that vaccination should be considered all the more if HAV itself is involved in the pathogenicity of hepatitis A, because safe and extremely effective inactivated HAV vaccines are available.

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INTERNAL MEDICINE

□ CASE REPORT □

Primitive Neuroectodermal Tumor as a Differential Diagnosis of CD56-Positive Tumors in Adults

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Abstract

A 33-year-old Japanese man was referred to our hospital after a huge intrapelvic tumor with bilateral hydronephrosis was found following persistent lumbago. Natural killer/T-cell lymphoma was suspected due to positive immunostaining for CD56, but CHOP therapy was ineffective. Re-evaluation of the tumor cells showed that they were positive for CD99, neuron-specific enolase, and synaptophysin and had a t(11 ; 22) (q 24 ; q12) translocation, leading to the revised diagnosis of primitive neuroectodermal tumor (PNET). Systemic chemotherapies and radiation therapy were added to surgical resection, and no recurrence has been detected for 3 years. Taken together, PNET may be considered in adult patients with CD56-positive tumors.

Key words: primitive neuroectodermal tumor, CD56, CD99, ICE-CAV therapy

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Introduction

Primitive neuroectodermal tumor (PNET) belongs to the Ewing sarcoma family and makes up approximately 1% of all sarcomas in Japanese children (1). Recently, it has been shown that a t(11 ; 22) (q24 ; q12) translocation (EWS/FLI-1 fusion) is essential for the development of Ewing sarcoma family tumors (ESFT) (2, 3). ESFT is relatively common as a cause of bone tumors in children, but is quite rare in adults (4, 5). Here, we report an adult case of PNET initially diagnosed as natural killer (NK)/T-cell lymphoma based on a positive result for CD56.

Case Report

A 33-year-old Japanese man was referred to our hospital for treatment of an intrapelvic tumor. He had suffered from multiple liver and gastric tumors at the age of 27. The tumor cells obtained from gastric mucosa had large round-shaped nuclei and were positive for CD20 (Fig. 1), but not for CD3. Since Epstein-Barr virus (EBV)-encoded small RNA (EBER) was detected in the nuclei of tumor cells by *in situ* hybridization (Fig. 1), he was diagnosed with EBV-related B-cell lymphoproliferative disorder. He was successfully treated with 6-time cyclophosphamide, adriamycin, vincristine, and prednisolone (CHOP) therapy and was in good health with no recurrence for 6 years after the treatment. However, he became worried about lumbago that persisted for two months and first visited another hospital, where he showed no signs of weight loss, fever, night sweats, or dysuria. Physical examination showed tenderness in bilateral costovertebral angles. Hepatosplenomegaly, swelling of superficial lymph nodes or tonsils, or an abnormal mass were not detected. Laboratory tests revealed increases in serum concentrations of urea nitrogen, creatinine, lactate dehydrogenase, β2-microglobulin, and soluble interleukin-2 receptor (Table 1), and an abdominal computed tomography (CT) scan revealed a huge tumor (10×10×6 cm) in the pelvis with bilateral hydronephrosis and swelling of the para-

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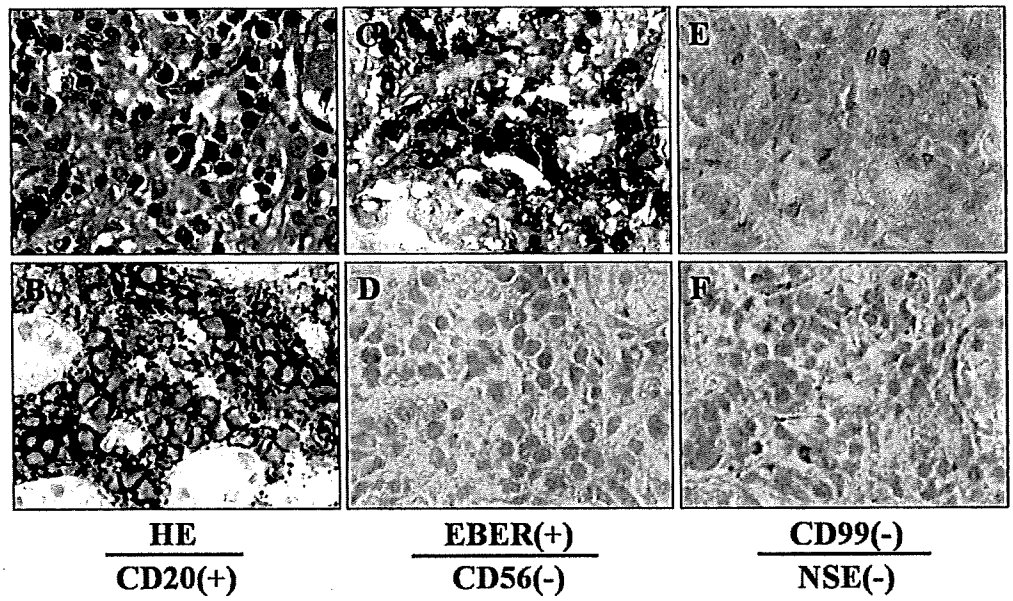

Figure 1. Histological findings of the gastric tumor that appeared 6 years ago. The tumor cells were relatively large, round-shaped [A: Hematoxylin and Eosin staining], and positive for immunostaining against CD20 (B) and *in situ* hybridization against EBV-encoded small RNA (EBER) (C). However, CD56 (D), CD99 (E) and NSE (F) were all negative.

Table 1. Laboratory Data on Admission

	Normal Range			Normal Range	
WBC (/μL)	3500-9000	6580	CRP (mg/dL)	<0.3	0.6
Hb (g/dL)	13-18	13.9	PT (%)	70-130	81.9
Platelet counts ($\times 10^3$/μL)	143-333	220	APTT (sec)	25-40	31.9
Albumin (g/dL)	3.8-5.3	3.6	Fibrinogen (mg/dL)	150-450	589.2
Bilirubin (mg/dL)	0.3-1.2	0.4	FDP (μg/dL)	<5	10.1
AST (IU/L)	13-33	12	FDP-D dimer (μg/dL)	<1	4.2
ALT (IU/L)	8-42	10	CEA (ng/mL)	<5	1.1
ALP (IU/L)	115-359	143	CA19-9 (U/mL)	<37	15.9
γGT (IU/L)	10-47	14	β2-MG (U/mL)	0.7-2.0	2.11
LDH (IU/L)	119-229	284	sIL2-R (mg/dL)	220-530	664
BUN (mg/dL)	8-22	48.1	anti EBV VCA IgM	<×10	<×10
Creatinine (mg/dL)	0.6-1.1	4.4	anti EBV VCA IgG	<×10	×320
UA (mg/dL)	3.6-7.0	6.3	anti EBV EBNA	<×10	×10
Na (mEq/L)	138-146	141	TPHA	(-)	(-)
K (mEq/L)	3.6-4.9	4.0	HBs antigen	(-)	(-)
Cl (mEq/L)	99-109	104	anti HCV antibody	(-)	(-)

Abnormal values were underlined. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGT, γ- glutamyltransferase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; UA, uric acid; CRP, C-reactive protein; PT, protoronbin time; APTT, activated partial thromboplastin time; FDP, fibrin and fibrinogen degradation product; β2-MG, beta2 microglobulin sIL2-R, soluble IL2 receptor; CEA, carcinoembryonic antigen; TPHA, *Treponema pallidum* latex agglutination; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; (-), negative.

aortic lymph nodes (Fig. 2). Intra-ureteral stents were inserted and a needle biopsy of the tumor was performed. The tumor cells were round-shaped and resembled the abnormal lymphoid cells obtained from his multiple gastric tumors 6 years earlier. These cells were also positive for CD56, but not for CD3 or CD20 (Fig. 2). Based on these results, a diagnosis of NK/T-cell lymphoma was made and the patient was transferred to our hospital for immediate chemotherapy.

According to the diagnosis of NK/T-cell lymphoma, CHOP therapy was commenced but did not improve the patient's lumbago or tumor. Re-examination of the needle biopsy specimen obtained at the previous hospital showed that the tumor cells were also positive for CD99, a hallmark of ESFT. In addition, a t(11 ; 22) (q24 ; q12) translocation (EWS/FLI-1 fusion) was detected by fluorescence *in situ* hybridization (FISH) (Fig. 3A) and reverse transcriptase-

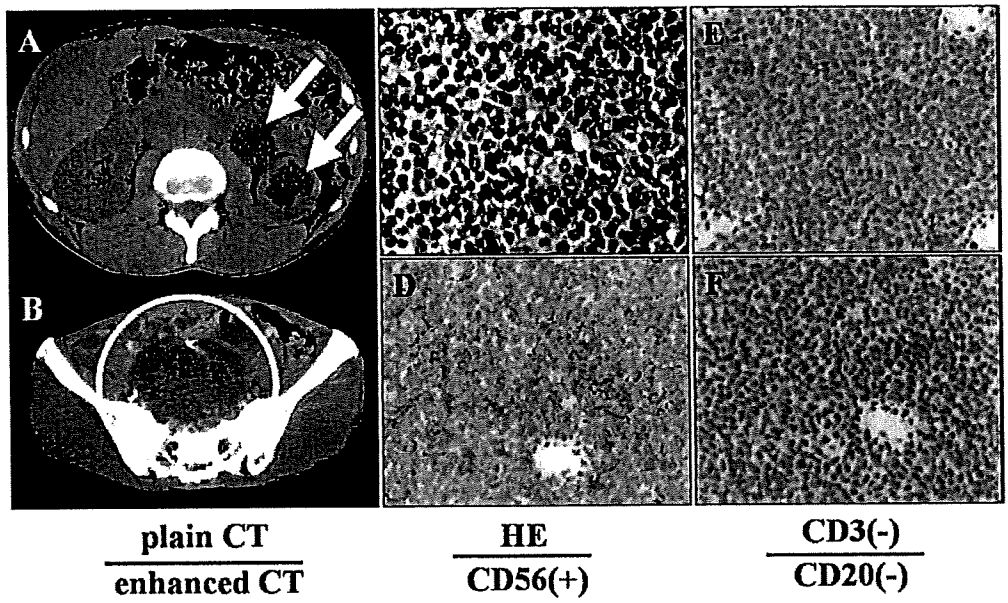

Figure 2. Radiological and histological findings of the intrapelvic tumor. (A) A plain abdominal CT scan showed hydronephrosis (arrows). (B) A contrast-enhanced abdominal CT scan revealed a huge tumor (circle). The tumor was heterogeneously stained by contrast medium. (C-F) Histological findings of the tumor specimen obtained by needle biopsy. The tumor cells were round-shaped [C, Hematoxylin and Eosin staining] and positive for immunostaining against CD56 (D). However, CD3 (E) and CD20 (F) were both negative.

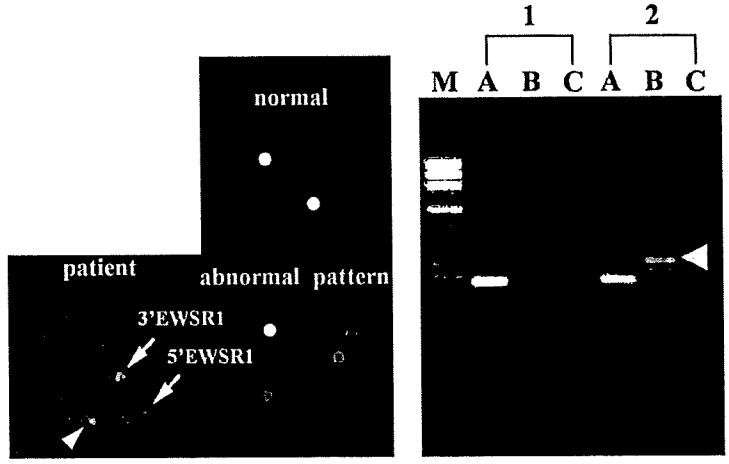

Figure 3. Detection of a t(11;22) (q24;q12) translocation (EWS/FLI-1 fusion). Fluorescence *in situ* hybridization (FISH) (left panel) and reverse transcriptase-polymerase chain reaction (RT-PCR) (right panel) were performed as described in Methods (6). In the FISH assay, the 3′- and 5′ regions of the EWSR1 gene were labeled as a green and red signal, respectively, and normal cells showed a 1-yellow fusion signal (arrowhead). The tumor cells demonstrated one pair of split red and green signals caused by translocation of the EWSR1 gene. In the right panel, the PCR products of β-actin (A), EWS/FLI-1 (B), and EWS/ERG (C) genes were subjected to electrophoresis. The presence of aberrant EWS/FLI-1 gene fusion transcripts was confirmed in the tumor (arrowhead). 1: normal subject; 2: this patient; M: molecular weight marker.

polymerase chain reaction (RT-PCR) (Fig. 3B) (6). Other histological markers suggestive of lymphoma or NK/T-cell lymphoma, such as CD45, CD34, terminal deoxynucleotidyl transferase (TdT), CD2, and EBER, were all negative. Based on these new findings, a revised diagnosis of intrapelvic ESFT was made. Since no distant metastasis was found, surgical removal of the tumor was attempted. Macroscopically, the tumor was dark red in color and smooth (Fig. 4). There

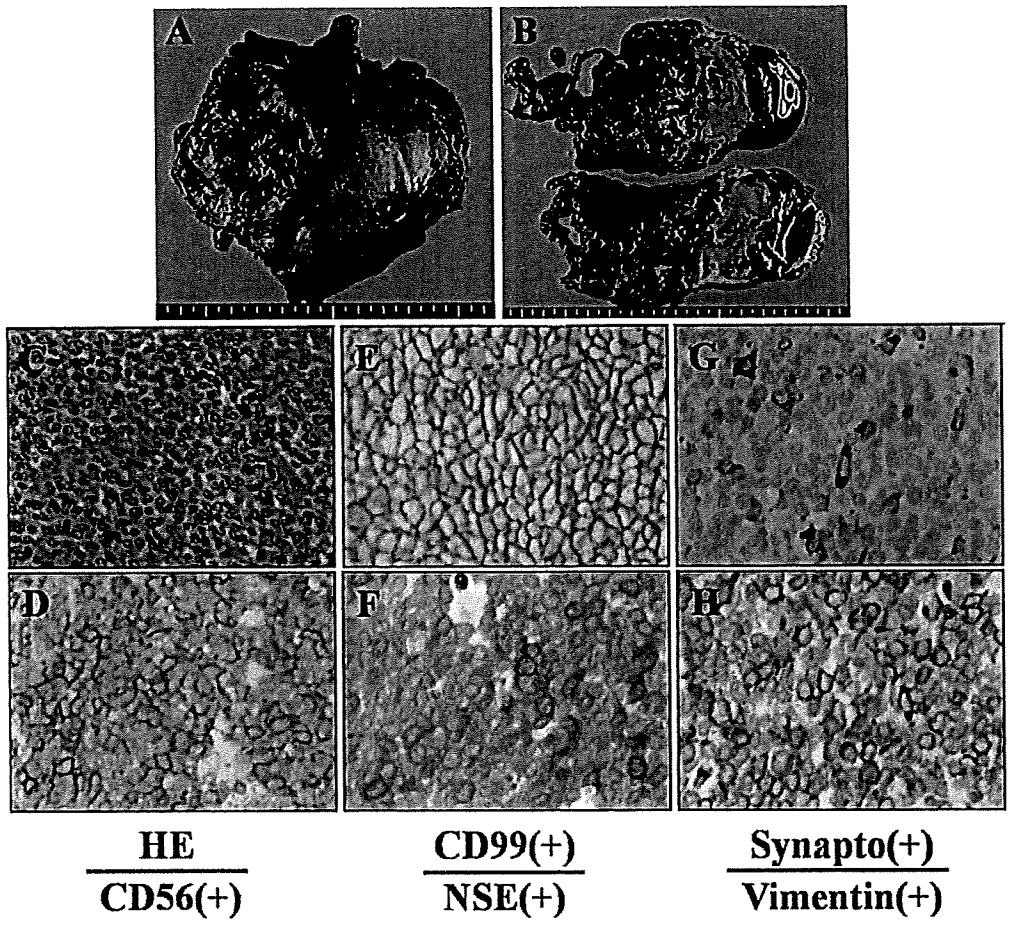

Figure 4. Macroscopic and microscopic findings of the resected tumor. (A) Gross appearance of the tumor (10×10×6cm). (B) Hemorrhage and necrosis were found in the tumor. (C-H) Histological findings of the resected tumor. The tumor cells partially formed pseudorosettes [C, Hematoxylin and Eosin staining]. The cells were positive for immunostaining against CD56 (D), CD99 (E), NSE (F), synaptophysin (G), and vimentin (H).

was no involvement of the iliac bones or lumbar vertebrae. The bilateral ureters were completely involved in the tumor. The tumor could be completely removed, but was seen to be partially adhered to the abdominal wall, suggesting the possibility of direct invasion. Histological sections stained with Hematoxylin and Eosin showed that the tumor cells were round-shaped and partially formed pseudorosettes. The cells were strongly positive for neuron-specific enolase (NSE), synaptophysin, and vimentin in addition to CD56 and CD99 (Fig. 4), which led to the final diagnosis of PNET. Considering the possibility of tumor residue, two types of systemic chemotherapies [ifosfamide, carboplatin, and etoposide (ICE) and cyclophosphamide, doxorubicine, and vincristine (CAV)] (Table 2) and regional radiation therapy were added after tumor resection. No serious adverse effects were observed during these treatments, and neither local recurrence nor distant metastasis has been found for over 3 years with careful monitoring.

Table 2. Regimens of ICE-VAD Therapy in This Case

ICE therapy		
Ifosfamide	1200 mg/m^2	days 1-5
Carboplatin	400 mg/m^2	day 1
Etoposide	100 mg/m^2	days 1-5
Mesna	1200 mg/m^2	days 1-5
CAV therapy		
Cyclophosphamaide	750 mg/m^2	days 1-5
Doxorubicin	75 mg/m^2/3days	days 1-3, continuous infusion
Vincristine	1.5 mg/m^2/3days	days 1-3, continuous infusion

Discussion

In Japan, ESFT, a generic disease term for Ewing sarcomas and PNET, is a relatively common sarcoma originating in the bones of patients younger than 20 years of age; approximately 80% of ESFT patients are children (4). As such, there are few reports regarding PNET in Japanese adults due to its rarity and difficulty in accurate pathological diagnosis.

Here, we report a rare case of adult PNET detected as a huge intrapelvic tumor.

In this case, the infrequency of PNET in adults and lack of careful histological examination delayed the correct diagnosis. The presence of a past history of EBV-related lymphoproliferative disorder and elevations in serum lactate dehydrogenase, β2-microglobulin, and soluble interleukin-2 receptor levels might have complicated the diagnosis as well. The patient's tumor cells were CD56-positive and CD3- and CD20-negative, so were initially diagnosed as NK/T-cell lymphoma. CD56, a neural cell adhesion molecule, is a hemophilic binding glycoprotein present on the surface of neurons, glia, skeletal muscle, and NK cells. Although CD56 has been established as a representative diagnostic marker of this type of lymphoma (7), it is also expressed in other non-hematological malignancies, such as olfactory neuroblastoma, neuroendocrine tumor, and alveolar rhabdomyosarcoma (8, 9). Thus, its positivity alone is insufficient to confirm the diagnosis of NK/T-cell lymphoma; if tumor cells are positive for CD56, additional immunostaining for other lymphocyte antigens, including CD45, CD34, and TdT, is required (10).

When NK/T-cell lymphoma can be ruled out in cases with a CD56-positive tumor, the possibility of ESFT should be taken into consideration. CD99 is often positive in ESFT (11), and other histological markers suggesting neuroectodermal differentiation, such as NSE, synaptophysin, and vimentin, may be positive as well, especially in PNET (12). Recently established genetic assays are also useful for clear differentiation between ESFT and other neurogenic tumors. For example, the t(11 ; 22) (q24 ; q12) translocation (EWS/FLI-1 fusion) is found in more than 85% of ESFT cases. Other translocations involving the EWS locus on chromosome 22, including t(21 ; 22) (q22 ; q12) and t(7 ; 22) (p22 ; p12) (EWS/ERG fusion), have also been identified (2, 3). Although EWS/FLI-1 and EWS/ERG fusions have been shown to promote the malignant transformation of cells (13), the precise function of fusion gene products remains to be elucidated.

The occurrence of a bulky mass or pelvic space, the presence of obvious metastasis, and advanced age are known as poor prognostic factors of adult ESFT (14-18). A retrospective study of 1,426 German ESFT patients demonstrated that an age of more than 15 years at the time of diagnosis and treatment elsewhere than in a pediatric oncology unit were associated with poor prognosis as well (19). The present patient had most of these unfavorable factors and the tumor was suspected to be directly invading the neighboring abdominal wall, which necessitated precautionary systemic chemotherapies and radiation therapy after surgical resection. Anti-cancer drugs, such as ifosfamide, etoposide, cyclophosphamide, vincristine, doxorubicin, carboplatin, and actinomycin D, have been proven to be effective for ESFT. Notably, the combination of ICE and CAV regimens has shown good results and is well-tolerated in adult patients with aggressive ESFT; the overall response rate to ICE-CAV therapy for advanced ESFT is reported to be 94%. Furthermore, it has also been shown that complete remission after ICE-CAV plus surgery is 95% and that the 3-year overall survival rate is estimated to be 67±12% (20).

The patient's negative results for CD56, CD99, and NSE in the abnormal cells of his EBV-related lymphoproliferative disorder, as evidenced by additional immunostaining, suggest a different strain from that of his PNET cells (Figs. 1, 4) and raises the possibility that the PNET in this case may have been a chemotherapy-associated secondary malignant neoplasm. In fact, a retrospective analysis of 11,183 patients who had undergone chemotherapies against primary malignant neoplasms during childhood or adolescence revealed a second malignancy in 479 patients. Of these, ESFT developed in 6 patients (21). Based on this report, the prevalence of ESFT after chemotherapy is estimated to be approximately 0.05%, which is considerably higher than that of idiopathic ESFT in the general population (0.0001-0.0002%). As far as we know, this is the first report indicating the possibility of ESFT as a second malignant neoplasm in the Japanese population. Accumulation of Japanese ESFT/PNET data will help clarify the novel aspect of ESFT/PNET as a second malignancy.

In conclusion, although ESFT/PNET is rare in adults, the possibility of this tumor should be kept in mind in patients with a CD56-positive tumor. The detection of CD99 expression and a t(11 ; 22) (q24 ; q12) translocation will yield the correct diagnosis of ESFT/PNET in such cases.

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Association analysis of Toll-like receptor 4 polymorphisms with autoimmune pancreatitis

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ABSTRACT

Autoimmune pancreatitis (AIP) is characterized by lymphoplasmocytic inflammation, high serum IgG4 concentrations, and a favorable response to corticosteroid treatment. Although long-term follow-up studies have shown that a relapse rate of 30–40% can occur in AIP after remission with corticosteroids, there are few genetic characteristic predictors of relapse in AIP patients. Toll-like receptor (TLR) is an important mediator in both innate and adaptive immunity. Polymorphisms in *TLR4* gene have been linked with several autoimmune and allergic diseases. We therefore investigated the genetic association between *TLR4* polymorphisms and AIP susceptibility and relapse in a Japanese population. Eight SNPs in *TLR4* (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953) were genotyped in 59 patients with AIP and 126 healthy controls using a TaqMan assay. Analysis of allelic frequencies revealed no statistical association with either susceptibility or relapse of AIP. These data indicate that *TLR4* polymorphisms do not play an important role in the development of AIP.

1. Introduction

Autoimmune pancreatitis (AIP) is characterized by irregular narrowing of the main pancreatic duct, swelling of the pancreas, histologic evidence of lymphoplasmocytic inflammation, and a favorable response to corticosteroid treatment [1–4]. We and others have previously reported that IgG4 concentrations are significantly and specifically higher in patients with AIP, suggesting that IgG4 plays a major role in AIP pathogenesis [5,6]. In addition, abundant IgG4-bearing plasma cells have been found infiltrating the pancreas in AIP [7,8]. This disease is also characterized by systemic complications involving various extra-pancreatic lymphoplasmocytic inflammation and IgG4-bearing plasma cell infiltration; thus AIP has been recognized as a systemic inflammatory condition [7–10]. Furthermore, we previously reported three susceptibility genetic markers [11–14]. However, because none of the genetic markers currently identified can sufficiently explain disease etiology, additional genes that influence immune tolerance are likely to be involved. Zen *et al.* recently reported that in patients with AIP, the Th2 and regulatory immune reactions were upregulated in the affected tissues [15]. These investigators indicated that the predominance of Th2 and regulatory immune reactions in AIP might reflect an allergic nature in the pathogenesis. According to recent studies on AIP, susceptibility and relapse of AIP are influenced by genetic factors, specific HLA alleles, amino acid sequences at the presentation site of the HLA molecule, and cytotoxic T-lymphocyte antigen 4 *(CTLA4)* SNPs [13,16].

Toll-like receptors (TLRs) are transmembrane proteins expressed by cells of the innate immune system, which recognize pathogen-associated molecular patterns and play important roles in immune and inflammatory responses to destroy the invaders. Among TLR family members, TLR4 (Toll-like receptor–4) has been the most thoroughly investigated. Apart from its involvement in the recognition of lipopolysaccharide, TLR4 also interacts with endogenous ligands, including heat-shock proteins. Some studies have reported that allergic diseases, including bronchial asthma and atopic dermatitis, are associated with single-nucleotide polymorphisms (SNPs) in the *TLR4* gene [17–19]. However, no study has comprehensively evaluated risk factors for AIP relapse and investigated the association between *TLR4* SNPs and AIP. Therefore, we examined the potential involvement of *TLR4* SNPs in the susceptibility and relapse of AIP.

2. Subjects and methods

2.1. Subjects

Between September 1994 and September 2007, we recruited 59 patients with AIP (49 men and 10 women), 38–76 years old (median, 63 years old), and 126 healthy control subjects. The diagnosis

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of AIP was based on criteria released by the Japan Pancreas Society using clinical data, imaging tests, and/or histopathologic findings, as reported previously [20]. Of the 59 patients with AIP, 37 (63%) had concurrent autoimmune diseases, including hypothyroidism (11 patients) and sclerosing cholangitis (34 patients); these diagnoses were described in prior studies [9,21]. All control subjects had indicated the absence of major illnesses on a standard questionnaire. This group was formed by enrolling volunteers from hospital staff. All racial/ethnic backgrounds were Japanese.

Serum levels of IgG4 were determined by single radial immunodiffusion kits (normal, <135 mg/dl) as reported previously [5]. High serum IgG4 concentrations (median, 730.0 mg/dl; interquartile range, 265.0–1037.5 mg/dl) were found in 55 of the 59 patients with AIP. Of the patients, 52 were treated with 40 mg prednisolone daily for 4 weeks; the dose was then reduced by 5 mg per week over a period of several weeks. All 52 patients responded favorably to corticosteroid therapy, resulting in improvements in clinical, laboratory, and imaging findings. We found no high concentrations of serum IgG4 in healthy subjects. All patients and controls were negative for the hepatitis B surface antigen and antibodies to hepatitis C in the serum [22].

In total, we followed the 55 patients with high IgG4 levels, including 52 patients who were treated with corticosteroids every month for a period of at least 12 months (median, 72 months; range, 12–178 months). Patients underwent regular follow-up visits with an interview every month; laboratory tests every 2–3 months, and imaging tests, including computed tomography or magnetic resonance imaging, every 6 months or, in the event of relapse, until September 2007. Of the 55 patients, 16 (29%) experienced relapse during follow-up. A relapse was defined as a recurrent attack of pancreatic swelling that resulted in irregular narrowing of the pancreatic duct or stenosis of the common bile duct, as reported previously [23].

All participants provided written informed consent for tests with DNA samples. After receiving permission, serum samples were obtained from patients and normal subjects. This study was approved by the institutional ethics committee.

2.2. TLR4 genotyping

Genomic DNA was isolated from whole blood of patients and healthy individuals using QuickGene-800 (Fujifilm, Tokyo, Japan). The concentration of genomic DNA was adjusted to 10–15 ng/μl for the TaqMan SNP genotyping assay. TLR4 is composed of four exons and has four transcript isoforms. We evaluated eight SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953) that were localized within the exons and introns of the *TLR4* gene. These SNPs were selected from among previous reports [24–26] and public information sources, such as the NCBI dbSNP (http://www.ncbi.nlm.nih.bov/SNP/), Applied Biosystems (http://www.appliedbiosystems.com), and HapMap (http://www.hapmap.org/) databses, and had minor allele frequencies >5%. The SNP spans approximately 1–5 kb, and includes 5 kb of the predicted 5′-untranslated region (UTR) and 6 kb of the predicted 3′ UTR in the *TLR4* gene. Genotyping of all SNPs was performed by a TaqMan 5′ exonuclease assay using primers supplied by (Applied Biosystems, Foster City, CA). The probe fluorescence signals were detected with a TaqMan Assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems), according to the manufacturer's instructions.

2.3. HLA typing

HLA class I and II alleles, and *DRB1* and *DQB1* alleles were identified, as reported previously [27,28]. These HLA typings had been done before, not for the purpose of this manuscript.

2.4. Statistical analysis

The Hardy-Weinberg equilibrium (HWE) test was done for each SNP among controls and patient groups. The pairwise linkage disequilibrium (LD) patterns, haplotype block structure, and haplotype frequency analysis for all SNPs were assessed by the block definition of Gabriel *et al.*, and was based on 95% CI of D′ with implementation of Haploview version 3.32 software [29,30] (http://www.broad.mit.edu/mpg/haploview/index.php). The significance of allele distribution between patients with AIP and healthy subjects was tested using the χ^2 test for 2 × 2 or 2 × 3 comparisons. When the number of subjects was less than 5, Fisher's exact test was used. A value of $p < 0.05$ was considered statistically significant. The corrected p value (p_c) was calculated by the Bonferroni's correction where the coefficient was the total number of the contingency tables tested.

3. Results

3.1. TLR4 genotyping in patients with AIP and healthy subjects

Eight SNPs in TLR4 were genotyped in 59 patients with AIP and in 126 healthy subjects (Table 1). In controls, the genotype distributions of all SNPs exhibited Hardy-Weinberg equilibrium, and the minor allele frequencies of all SNPs were more than 5%. However, in patients, the genotype distribution of one SNP (rs2149356) differed significantly from the expected Hardy-Weinberg values ($p < 0.05$) (Table 1). All eight SNPs were located in 1 haplotype block, and the magnitude of LD between each SNP was high (Fig. 1). Analysis of allelic frequencies (Table 2) revealed a significant difference between patients with AIP and healthy subjects for SNP rs2149356: Positivity for G was significantly higher in patients with AIP than in healthy subjects ($\chi^2 = 8.58$, $p = 0.014$). The G/T genotype was significantly increased in patients with AIP compared with healthy subjects. This SNP preliminary showing statistical significance was later confirmed as not significant after correction for multiple testing. No other SNPs were significantly associated with AIP. The statistical power of this study was 0.9349 and enough for analysis.

Table 1
Allele frequencies of SNPs of the TLR4 gene in AIP patients and controls

dbSNP	Alleles (1/2)	Position (bp)	Gene location	Patients ($n = 59$)		Controls ($n = 126$)	
				MAF (%)	HWE	MAF (%)	HWE
rs10759930	T/C	119,501,442	5′-UTR	38.1	0.276	33.3	0.801
rs1927914	A/G	119,504,546	5′-UTR	38.1	0.276	32.9	0.698
rs1927911	G/A	119,509,875	Intron	35.6	0.629	32.9	0.235
rs12377632	C/T	119,512,551	Intron	35.6	0.629	32.5	0.602
rs2149356	G/T	119,514,020	Intron	31.4	0.007	32.1	0.511
rs11536889	G/C	119,517,952	3′-UTR	26.3	1.000	26.2	1.000
rs7037117	A/G	119,523,484	3′-UTR	20.3	0.505	17.5	0.282
rs7045953	A/G	119,525,616	3′-UTR	10.2	1.000	7.5	0.961

1, major allele; 2, minor allele; bp, base pair.
Position is distance from short arm telomere.

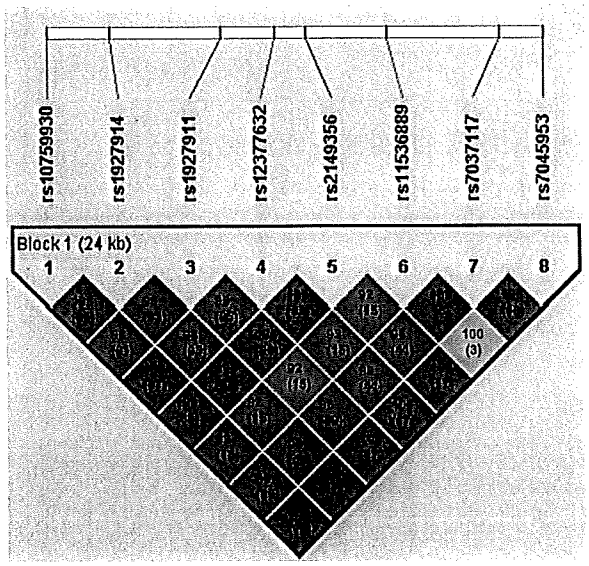

Fig. 1. Structure of linkage disequilibrium (LD) plot of 8 SNPs of the *TLR4* gene in the controls. The D' value and r^2 value (in parentheses) corresponding to each SNP pair are express as a percentage and shown within the respective square. Higher D' is indicated by a brighter red. The 8 SNPs constitute a haplotype block spanning 24 kb of the *TLR4* gene.

The haplotype frequency of the eight SNPs was estimated with the expectation-maximization algorithm. Nine unique SNP haplotypes were found altogether, and five had frequencies greater than 5% (Table 3). Association analysis using haplotypes calculated by expectation-maximization algorithms showed none of haplotypes were associated with either susceptibility or resistance to AIP.

We previously reported that the *HLA DRB1**0405-*DQB1**0401 haplotype was associated with AIP [11,12]; thus, we further investigated the genetic association between HLA haplotype and *TLR4* SNPs in patients with AIP. Analysis of allelic frequencies revealed no significant difference in SNP5 ($\chi^2 = 0.52$, $p = 0.77$) between patients with and without the *HLA DRB1**0405-*DQB1**0401 haplotype.

3.2. Associations among TLR4 SNPs, patient characteristics, and AIP relapse

Next, we examined associations between the *TLR4* SNPs and clinical parameters. We found no associations between any of the eight *TLR4* SNPs and age, gender, or serum IgG4 concentrations (data not shown). In particular, the median serum IgG4 concentration was not significantly different between patients with and without the SNP5G allele (730 vs 728 mg/dl; $p = 0.94$).

Previous studies found that AIP was associated with the autoimmune diseases, sclerosing cholangitis (34/44; 77%) and hypothyroidism (11/50; 22%) [13]. Thus, we evaluated whether sclerosing cholangitis or hypothyroidism were associated with any of the eight *TLR4* SNPs. We found no significant association between the *TLR4* SNPs and the two diseases (data not shown). Other studies found an association between relapse of AIP and genetics [13,19]; thus, we further analyzed the relationship between *TLR4* SNPs and risk of AIP relapse. In our cohort, 16 of 55 patients (29%) experienced relapse during the follow-up period. However, we found no significant associations between the SNPs or the haplotypes and the relapse of AIP.

4. Discussion

In previous studies, we determined that the *HLA DRB1**0405-*DQB1**0401 haplotype correlated with an increased prevalence of AIP in the Japanese population [11,12]. However, none of the previously identified genetic markers were sufficient to fully explain the disease etiology. Therefore, we suspected that a number of genes outside the major histocompatibility complex region might play a role in AIP susceptibility. For instance, we previously identified polymorphisms of the *FCRL3* and *CTLA4* genes that correlated with an increased prevalence of AIP in the Japanese population [13,14]. However, these findings have not been examined and confirmed in other ethnicities. *TLR4* is an interesting candidate for a gene related to AIP susceptibility because it has previously been implicated in other autoimmune diseases and allergic diseases, including rheumatoid arthritis, Behçet's disease, bronchical asthma, and atopic dermatitis [16–18,31,32].

Of the two co-segregating missense mutations in the gene encoding *TLR4*, A896G and C1196T (which result in Asp299Gly and Thr399Ile amino acid changes, respectively), only A896G interrupted TLR-4 signaling [10]. Most studies that reported disease associations with *TLR4* SNPs have shown significantly higher frequencies of SNPs related to the A896G and C1196T mutations. However, we did not detect polymorphisms on these two SNPs in 100 Japanese healthy controls, consistent with other reports including HapMap data. In the present study, we examined eight SNPs. Only rs2149356 SNP was statistically associated with AIP before correction of the *p* value. However, this SNP, among all SNPs

Table 2
TLR4 polymorphisms in 59 patients with AIP and 126 healthy subjects

dBSNP	Frequency (%)		*p*	OR	95% CI
	AIP ($n = 59$)	Controls ($n = 126$)			
rs10759930					
Allele frequency					
C allele	38.1	33.3	0.37	1.24	0.78–1.94
T allele	61.9	66.7			
rs1927914					
Allele frequency					
G allele	38.1	32.9	0.33	1.26	0.80–1.98
A allele	61.9	67.1			
rs1927911					
Allele frequency					
A allele	35.6	32.9	0.61	1.13	0.71–1.78
G allele	64.4	67.1			
rs12377632					
Allele frequency					
C allele	64.4	67.5	0.57	1.15	0.72–1.81
T allele	35.6	32.5			
rs2149356					
Genotype frequency					
G/G	39.0	47.6	0.014*		
G/T	59.3	40.5			
T/T	1.7	11.9			
Allele carrier frequency					
G (G/G+G/T)	98.3	88.1	0.021**	7.84	1.01–60.82
T (G/T+T/T)	61.0	52.4	0.27	1.43	0.76–2.67
Allele frequency					
G allele	68.6	67.9	0.98	1.04	0.65–1.66
T allele	31.4	32.1			
rs11536889					
Allele frequency					
A allele	26.3	26.2	0.91	1.00	0.61–1.65
G allele	73.7	73.8			
rs7037117					
Allele frequency					
A allele	79.7	82.5	0.60	1.21	0.69–2.10
G allele	20.3	17.5			
rs7045953					
Allele frequency					
G allele	89.8	92.5	0.52	1.39	0.65–2.96
T allele	10.2	7.5			

AIP, autoimmune pancreatitis; OR, odds ratio; 95% CI, 95% confidence interval.
p Value was calculated by χ^2 test 2 × 2 contingency table (df = 1), or test 3 × 2 contingency table (df = 2).
*p_c (corrected *p* value) = 0.042, **$p_c = 0.17$.

Table 3
TLR4 haplotypes in patients with AIP and healthy subjects

Haplotype	8 SNPs within a haplotype block spanning 24 Kb								Proportion of indicated haplotype (%)		p value
	1 rs10759930	2 rs1927914	3 rs1927911	4 rs12377632	5 rs2149356	6 rs11536889	7 rs7037117	8 rs7045953	AIP ($n = 118$)	Controls ($n = 252$)	
HP1	T	A	G	C	G	G	A	A	33.9	41.4	0.21
HP2	T	A	G	C	G	C	A	A	28.0	24.0	0.43
HP3	C	G	A	T	T	G	A	A	11.0	15.1	0.32
HP4	C	G	A	T	T	G	G	A	10.2	9.5	0.85
HP5	C	G	A	T	T	G	G	G	10.2	6.9	0.26

AIP, autoimmune pancreatitis.
Values for n indicate two times the number of individuals since each person carries two haplotypes.

tested in the patients group, significantly deviated from HWE in the patient group. The deviation might be explained by collecting samples of affected individuals selectively, because HWE failure is generally caused by migration, mutation, gene flow, genetic drift, nonrandom mating, and natural selection. This phenomenon is thought to be the cause of decreasing homozygous TT and GG carriers in contrast to increasing heterozygous TG carriers in the patients. The functional effects of increased TG heterozygotes in patients are still uncertain. However, as there were only a small number of patients in our cohort, it will be necessary to confirm this association in future studies with larger cohorts. Although the statistical power was enough as 0.9349 in this study, type II error was also a possible explanation for the lack of association between TLR4 SNPs and AIP.

Although previous work has shown that serum IgG4 concentrations were closely associated with AIP, we found no significant correlations between serum IgG4 concentrations and *TLR4* SNPs or haplotypes in this study. In addition, our analysis of patients with AIP that also had sclerosing cholangitis and hypothyroidism indicated that there were no associations between extrapancreatic complications and *TLR4* SNPs or haplotypes.

We checked whether the *HLA DRB1**0405-*DQB1**0401 haplotype and rs2149356 SNP was independently associated with AIP. However, we found no confounding association between the *HLA DRB1**0405-*DQB1**0401 haplotype and rs2149356 SNP. This result was similar to that shown in a previous study that found no association between the *HLA DRB1**0405-*DQB1**0401* haplotype and *FCRL3*-110 alleles, although each was independently associated with AIP [14].

Long-term follow-up studies have shown that a 30-40% rate of AIP relapse can occur after remission with corticosteroids [13,19,23,33]. However, no characteristic risk factors or predictive markers have been identified that might be associated with a relapse of AIP. We recently found that patients that experienced relapse had high associations with *CTLA4* +49A/A or +6230A/A genotypes [13]. Furthermore, substitution of aspartate to a non-aspartate residue at HLA DQβ1 57 was associated with a relapse of AIP in a Korean cohort [19]. In the present study, we examined whether any of the *TLR4* SNPs were associated with an AIP relapse but found no associations. Further studies are required to identify predictive markers of AIP relapse.

In conclusion, we found that *TLR4* gene polymorphisms were not significantly associated with susceptibility to AIP in Japan. However, the connection between genetic variations and susceptibility to AIP and AIP relapse remains to be addressed in future investigations.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Nonalcoholic fatty liver disease in Japanese junior high school students: its prevalence and relationship to lifestyle habits

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Abstract
Background Despite the increase in nonalcoholic fatty liver disease (NAFLD) in Japanese adults, its prevalence in adolescents remains unclear. This prompted us to evaluate the incidence and clinical characteristics of NAFLD among junior high school students.
Methods A population-based cross-sectional study was conducted among students in a single junior high school in Nagano prefecture. Serum alanine aminotransferase (ALT) and γ-glutamyltransferase (γGT) measurements and abdominal ultrasonography were performed in 249 and 288 students in 2004 and 2007, respectively. In the latter survey, student lifestyle habits were also assessed, using questionnaires.
Results The prevalence of NAFLD was 4.4% and 4.5% in 2004 and 2007, respectively, which was lower than that of obesity (10.0% and 5.9%). Body mass index and ALT and γGT levels increased significantly with hepatic steatosis severity. Multivariate logistic regression analysis demonstrated that the presence of obesity and an ALT level of 30 U/L or more were independent predictors of NAFLD (odds ratio 16.9, $P < 0.001$ and odds ratio 16.6, $P = 0.001$, respectively). The ratios of students commuting to and from school by car and not doing sports outside of school were higher in NAFLD students compared with non-NAFLD ones. Such tendencies were observed independently of the presence of obesity. Additionally, one obese student with severe steatosis and liver dysfunction was diagnosed as having nonalcoholic steatohepatitis (NASH).
Conclusions Approximately 4% of junior high school students had NAFLD that was primarily associated with obesity and reduced daily physical activity. Serum ALT measurement during school check-ups is recommended for the early detection of young adolescent NAFLD/NASH.

Keywords Obesity · ALT · Physical activity · Skipping breakfast · Nonalcoholic steatohepatitis

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Introduction

Due to increasing sedentary lifestyles and the rising prevalence of obesity, nonalcoholic fatty liver disease (NAFLD) has become a common cause of chronic liver disease. NAFLD encompasses a spectrum of histological findings that range from macrovesicular steatosis alone (simple

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steatosis) to macrovesicular steatosis with hepatocyte ballooning and/or lobular inflammation (steatohepatitis). Non-alcoholic steatohepatitis (NASH) is the severe and progressive form of NAFLD and may develop into cirrhosis, hepatocellular carcinoma, and ultimately death [1–3]. Based on current health screening data, the prevalence of NAFLD in Japanese adults is estimated to be around 10% [1].

Of great recent concern is the fact that NAFLD/NASH exists even in children and adolescents and that pediatric NASH can also progress to cirrhosis. Tominaga et al. [4] reported that 2.6% of Japanese children aged 4–12 years had NAFLD. Furthermore, Kinugasa et al. [5] described seven Japanese obese children aged between 9 and 15 years having hepatic steatosis with various degrees of lobular inflammation and/or portal fibrosis and one child having cirrhosis. Therefore, the early detection of NAFLD/NASH and appropriate disease management are now important in the pediatric population as well.

According to data from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the prevalence of obesity among Japanese junior high school students was double in 2003 compared to that in 1977 (from 5% to 10%). Given the strong association of NAFLD with obesity, NAFLD is suspected to affect a substantial, but as yet unidentified, proportion of junior high school students. As such, this population-based cross-sectional study was planned to evaluate the prevalence of NAFLD in young adolescents, along with any lifestyle habits associated with its development.

Materials and methods

Participants

The study population consisted of all students attending a public junior high school in a village located in southern Nagano prefecture. Real-time liver function tests and abdominal ultrasonography (US) were performed in 2004 and 2007 after explaining the significance and protocol of the study to all students and their parents and obtaining written informed consent. This study was approved by the ethics committee of Shinshu University School of Medicine and Showa Inan General Hospital and adheres to the principles of the Declaration of Helsinki. In this study, NAFLD was defined by the existence of hepatic steatosis based on abdominal US, regardless of liver function tests.

Data collection

Anthropometric, biochemical, and ultrasonographic examinations were carried out on the same school-day mornings in June 2004 and July 2007 after an overnight fast. Body height and weight were measured by a school nurse, with subjects barefoot and in light clothing. Body mass index (BMI) and the age-gender-adjusted Japanese standardized weight index for height (JSI) were used as anthropometric parameters. The JSI was calculated as [(body weight − standard body weight)/standard body weight] × 100 (%), where the standard body weight for each subject's age, sex, and height was determined from data on 700 000 Japanese children aged 5–17 years in 1990. The JSI is considered to be more suitable for the evaluation of the physiques of children and adolescents than the BMI in Japan. According to the JSI, subject weight status was classified as lean ($\leq -20\%$ of JSI), moderately lean (-19.9% to -10.1%), normal (-10% to $+10\%$), overweight ($+10.1\%$ to $+19.9\%$), or obese ($\geq +20\%$).

Venous blood samples were drawn just after an anthropometric examination. Serum levels of alanine aminotransferase (ALT), γ-glutamyltransferase (γGT), triglycerides (TG), and high-density-lipoprotein cholesterol (HDL-C) were determined using standard automated analyzers. Normal ranges for serum ALT and γGT levels were set as 0–30 U/L each, in accordance with previous studies in pediatric populations [6–8].

Real-time abdominal US was performed by two experienced ultrasonographers (K.H. and C.I.) using a LOGIQ book equipped with a 4.0 MHz convex-type transducer (GE Yokogawa Medical Systems, Tokyo, Japan). Representative US images of each student were kept in the LOGIQ book and evaluated afterwards in a blinded manner by three independent hepatologists (G.T., N.T., and M.K.). The degrees of hepatorenal contrast, profound attenuation of the diaphragm, and blurring of the vascular wall were each scored as 0 (absent), 1 (present), or 2 (marked) [4, 9–11] The sum of these scores from each diagnostician ranged from 0 to 6, and a mean total score of 1 or more was judged as the presence of hepatic steatosis. Total scores of 1–2 and 3 or more were classified as the presence of mild steatosis and moderate-to-severe steatosis, respectively.

Assessment of lifestyle habits

In the 2007 survey, lifestyle habits were assessed in addition to abdominal US and liver function tests. A 15-item questionnaire focusing on personal dietary and exercise habits was prepared and distributed to all students. All questionnaires were completed and submitted by the students themselves, and were then analyzed for any relationships with NAFLD.

Statistics

Statistical analyses were performed using SPSS software 11.0J for Windows (SPSS, Chicago, IL, USA). Qualitative

variables were expressed as numbers (percentages) and compared using the χ^2 test. Quantitative data were expressed as means ± SD and compared using the two-tailed Student's *t* test or one-way analysis of variance. Post-hoc comparison was also performed between groups, using the Tukey's or Games–Howell's method. Multivariate logistic regression analysis was conducted to find independent predictors of NAFLD. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Prevalence of obesity and NAFLD

The overall prevalence of obesity was 10.0% and 5.9% in 2004 and 2007, respectively (Table 1). Because all students were presumed to not habitually consume alcohol, the overall prevalence of NAFLD was calculated as 4.4% and 4.5% in 2004 and 2007, respectively (Table 1). Of these, the prevalence of moderate-to-severe steatosis was 0.8% in 2004 and 1.4% in 2007 (Table 1). There was a male preponderance in NAFLD prevalence in the 2004 survey (7.5% in boys vs. 1.6% in girls, $P = 0.022$).

Association between weight status and NAFLD

As shown in Table 2, approximately 90% of students had neither obesity nor NAFLD and 2%–3% had both disorders. In the 2007 survey, 4 of 13 NAFLD students were overweight (Table 2). Although a small number of NAFLD students having normal body weight was found (1.2% in 2004 and 1.0% in 2007), the degree of steatosis was very mild in all cases. On the other hand, all students with moderate-to-severe steatosis were obese (Table 2).

Comparison of clinical data between students with and without NAFLD

In both surveys combined, 24 (4.5%) of the 537 students were judged as having NAFLD. Several parameters were then compared between students with NAFLD ($n = 24$) and those without ($n = 513$) to investigate the clinical features of NAFLD. The prevalence of male gender, obesity, and elevated ALT and γGT levels was significantly higher in the NAFLD group than in the non-NAFLD one (Table 3). JSI, BMI, and serum levels of ALT, γGT, and TG were increased and serum HDL-C levels were decreased in the NAFLD group (Table 3). When these parameters were analyzed in relation to the degree of steatosis, BMI and serum levels of ALT, γGT, and TG all increased with the steatosis severity (Fig. 1). Multivariate logistic regression analysis revealed that the presence of obesity and an ALT level of 30 U/L or more were independent predictors of NAFLD. The odds ratio was 16.9 for the presence of obesity (95% confidence interval [CI], 6.5–43.9; $P < 0.001$) and 16.6 for an ALT level of of 30 U/L or more (95% CI, 3.1–87.6; $P = 0.001$).

Additionally, when these parameters were compared between boys ($n = 275$) and girls ($n = 262$), JSI and serum levels of ALT and γGT were increased in the former group (2.6 ± 17.2 vs. −1.5 ± 14.2, $P = 0.003$ for JSI;

Table 1 Prevalence of obesity and NAFLD

Year	2004			2007		
	Boys ($n = 120$)	Girls ($n = 129$)	Overall ($n = 249$)	Boys ($n = 155$)	Girls ($n = 133$)	Overall ($n = 288$)
Weight status						
Lean	2 (1.7)	2 (1.5)	4 (1.6)	0 (0)	13 (9.8)	13 (4.5)
Moderately lean	9 (7.5)	21 (16.3)	30 (12.0)	23 (14.8)	38 (28.6)	61 (21.2)
Normal	87 (72.5)	81 (62.8)	168 (67.5)	102 (65.8)	61 (45.9)	163 (56.6)
Overweight	9 (7.5)	13 (10.1)	22 (8.8)	19 (12.3)	15 (11.3)	34 (11.8)
Obese	13 (10.8)	12 (9.3)	25 (10.0)	11 (7.1)	6 (4.5)	17 (5.9)
JSI (%)	2.9 ± 15.1	1.9 ± 14.3	2.4 ± 14.7	2.4 ± 18.7	−4.7 ± 13.4	−0.9 ± 16.8
BMI (kg/m^2)	19.7 ± 3.0	19.9 ± 3.0	19.8 ± 3.0	19.2 ± 2.7	19.2 ± 2.8	19.2 ± 2.7
Hepatic steatosis						
Absent	111 (92.5)	127 (98.4)	238 (95.6)	147 (94.8)	128 (96.2)	275 (95.5)
Present (NAFLD)	9 (7.5)	2 (1.6)	11 (4.4)	8 (5.2)	5 (3.8)	13 (4.5)
Mild	8 (6.7)	1 (0.8)	9 (3.6)	4 (2.6)	5 (3.8)	9 (3.1)
Moderate-to-severe	1 (0.8)	1 (0.8)	2 (0.8)	4 (2.6)	0 (0)	4 (1.4)

Data are expressed as numbers (percentages of students of the same gender or overall in each year) or means ± SD. Body weight status was classified according to the criteria of the age-gender-adjusted Japanese standardized weight index for height (JSI)

BMI body mass index, *NAFLD* nonalcoholic fatty liver disease