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Table 1. Patient characteristics.

No. of patients		312
Age at diagnosis, years, median (range)		8 (1-17)
Gender	male / female	186 /126
Etiology	no. of patients (%)	
Idiopathic		261 (83.7)
Hepatitis		44 (14.1)
Others		7 (2.2)
Severity of AA	no. of patients (%)	
VSAA		156 (50.0)
SAA		107 (34.3)
MAA		49 (15.7)
Peripheral blood data at diagnosis		
Median WBC count, $\times 10^9$/L (range)		2.02 (0.20-8.70)
Median neutrophil count, $\times 10^9$/L (range)		0.22 (0.00-3.13)
Median lymphocyte count, $\times 10^9$/L (range)		1.82 (0.10-8.50)
Median Hb level, g/dl (range)		6.9 (2.1-13.2)
Median reticulocyte count, $\times 10^9$/L (range)		16.0 (0.0-98.0)
Median platelet count, $\times 10^9$/L (range)		11.0 (1.0-109.0)
Interval from diagnosis to treatment, days, median (range)		15 (1-180)

VSAA indicates very severe aplastic anemia; SAA, severe aplastic anemia; MAA, moderate aplastic anemia; WBC, white blood cell; Hb, hemoglobin.

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Table 2. Univariate and multivariate analysis for IST response in 312 patients with AA.

Univariate variables	Responder	Non-responder	*P*
No. of patients (%)	176 (56.4)	136 (43.6)	
Median age at diagnosis, y	8	8	NS
Gender, male / female	115/61	71/65	0.025
Etiology, no. of patients (%)			
Idiopathic	141(80)	120(88)	NS
Hepatitis	29(17)	15(11)	
Others	6(3)	1(1)	
Severity of AA, no. of patients (%)			
VSAA	90(51)	66(49)	NS
SAA	62(35)	45(33)	
MAA	24(14)	25(18)	
Median WBC count, $\times 10^9$/L	1.900	2.255	0.007
$\geq 2.0\times 10^9$/L , no. of patients (%)	87(47)	85(63)	0.009
$< 2.0\times 10^9$/L , no. of patients (%)	93(53)	51(37)	
Median lymphocyte count, $\times 10^9$/L	1.600	2.016	0.006
Median neutrophil count, $\times 10^9$/L	0.218	0.200	NS
Median Hb level, g/dl	6.8	6.8	NS
Median reticulocyte count, $\times 10^9$/L	15.730	17.600	NS
Median platelet count, $\times 10^9$/L	10.000	11.000	NS
Interval from diagnosis to treatment, d	13	19	0.002
Multivariate variables	**Odds ratio**	**95% CI**	***P***
WBC count, $< 2.0\times 10^9$/L	3.219	1.707-6.070	0.0003
Interval from diagnosis to treatment, < 30 d	2.571	1.225-5.396	0.012
Gender, male	1.873	1.042-3.366	0.036
Reticulocyte count, $>25\times 10^9$/L	1.589	0.843-2.997	NS
Platelet count, $>20\times 10^9$/L	1.362	0.657-2.826	NS
Etiology, hepatitis/others	1.223	0.504-2.966	NS

VSAA indicates very severe aplastic anemia; SAA, severe aplastic anemia; MAA, moderate aplastic anemia; WBC, white blood cell; Hb, hemoglobin.

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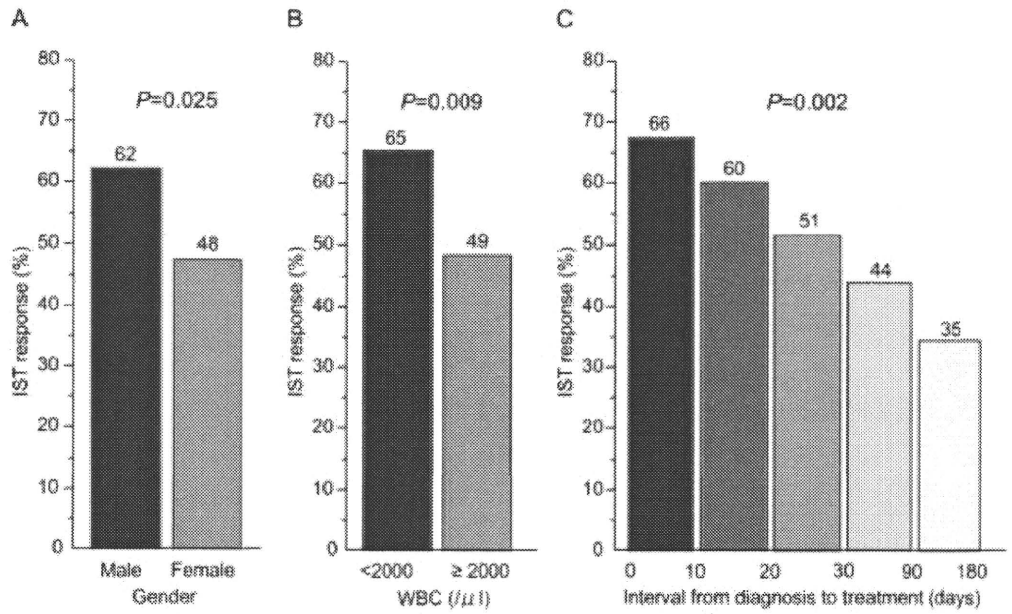
Figure legends

Figure 1. Response to IST in 312 patients according to WBC count, gender, and interval from diagnosis to treatment.

(A) Response rate according to gender. Boys showed better response than girls (62% vs. 48%, respectively; *P*=0.025). (B) Response rate according to WBC counts. Patients with WBC count <2.0×10^9/L displayed a significantly higher response rate than patients with WBC ≥2.0 ×10^9/L (65% vs. 49%, respectively; *P*=0.009). (C) Response rate according to the interval between diagnosis and treatment. Response rate was inversely associated with the interval between diagnosis and treatment (*P*=0.002).



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Indirect immunohistochemical evaluation of graft fibrosis and interface hepatitis after pediatric liver transplantation

Nagai S, Ito M, Kamei H, Nakamura T, Ando H, Kiuchi T. Indirect immunohistochemical evaluation of graft fibrosis and interface hepatitis after pediatric liver transplantation.
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Abstract: Fibrosis or IH following pediatric liver transplantation is recognized as major causes of graft loss, but the etiology remains unclear. To determine this issue, we used an indirect immunohistochemistry technique with post-transplant serum samples from recipients and normal human liver tissues from living liver donors, and the association between occult antibody reaction to the liver and the occurrence of fibrosis or IH was evaluated. Forty-three recipients were evaluated, and both hepatocytes and biliary epithelial cells were evaluated for staining intensity. Fibrosis and IH occurred in 13 and six patients, respectively. According to staining results for the hepatocytes and biliary epithelial cells, 18 and 11 patients, respectively, were classified into the positive group. According to log-rank analysis, positive reaction for hepatocytes was associated with increased rates of fibrosis and IH ($p = 0.002$ and 0.048, respectively), while positive reaction for biliary epithelial cells was associated with an increased rate of fibrosis ($p = 0.014$). Multivariate analysis revealed that positive reaction for hepatocytes and biliary epithelial cells was independently associated with fibrosis occurrence ($p = 0.020$ and 0.047, respectively). In conclusion, immune-mediated reactions by occult antibodies may underlie the pathogeneses of fibrosis and IH.

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Key words: liver transplantation – fibrosis – interface hepatitis – immunohistochemistry – late cellular rejection – *de novo* autoimmune hepatitis

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Liver transplant recipients often have abnormal histology findings during long-term follow-up, which may lead to late-onset graft dysfunction (1–8). There are several causes of late-onset graft dysfunction, such as vascular and biliary complications, recurrent primary disease, and infections, and more recently *de novo* AIH and fibrosis have been noted (4–13). *De novo* AIH is characterized by IH, along with the presence of autoantibodies and hypergammaglobulinemia (14–16). The etiology is uncertain, although it is considered to be a kind of rejection (6, 7). Moreover, it was reported that pediatric liver transplant recipients sometimes show chronic hepatitis, which is predominantly portal-based mononuclear inflammation associated with IH, which does not meet the criteria of *de novo* AIH (3, 5). These pathological changes are also considered to lead to graft dysfunction. In contrast, fibrosis is a consequence of a variety of complications, including vascular and biliary complications, viral hepatitis, and immunological morbidity, such as intractable acute cellular rejection, chronic ductopenic rejection, and occult chronic hepatitis (17). Fibrosis sometimes develops without obvious clinical signs, although it was reported that the presence of

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANA, antinuclear antibodies; ASMA, anti-smooth muscle antibody; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; IgG, immunoglobulin G; IH, interface hepatitis; LKM-1, anti-liver kidney microsomal type 1; TB, total bilirubin.

autoantibodies is a risk factor for fibrosis development (1, 3, 5, 9, 12, 13).

We previously reported that recipient serum often contains antibodies against donor T lymphocytes (18, 19), and speculated that occult antibodies in that serum provoke immune reactions to the liver graft, possibly leading to late graft dysfunction. In the present study, we utilized an indirect immunohistochemistry technique to test recipient serum samples. Our method is based on the theory that indirect immunohistochemistry is able to detect unknown antibodies (20, 21), as it has been proposed that immunological reactions of antibodies produced in recipient serum against a normal human liver can be detected (22). Using this method, we investigated the relationship between positive reactions and late-onset graft morbidity.

Materials and methods

Forty-three patients (15 males, 28 females) who underwent living donor liver transplantation between 1992 and 2008 were evaluated. All patients underwent liver biopsy at least once after the LT. Table 1 shows patient's characteristics. Only one patient was an adult (25.3 yr old), but primary diagnosis is biliary atresia. Therefore, this patient was included in this study. Informed consent was obtained from each patient or his/her parents before study entry.

Table 1. Patient characteristics

Patient present age (yr)	11.0 ± 6.5 (median, 8.6; 2.3–27.1)
Patient age at LT (yr)	4.8 ± 5.7 (median, 2.0; 4 months to 25.3)
Gender	
Male	15 (35%)
Female	28 (65%)
Follow-up period (yr)	6.2 ± 3.6 (median, 5.2; 1.1–15.8)
Indication for LT	
Biliary atresia	31 (72%)
Fulminant hepatic failure	6 (14%)
Metabolic disease	4 (9%)
Metastatic solid and cystic tumor of the pancreas	1 (2%)
Congenital extrahepatic portal venous obstruction	1 (2%)
Graft type	
Left lateral segment	32 (74%)
Monosegment	3 (7%)
Left liver	4 (9%)
Right liver	4 (9%)
Blood type compatibility	
Identical	29 (67%)
Compatible	10 (23%)
Incompatible	4 (9%)*

LT, liver transplantation.
*One of four patients was more than one yr old, and additional immunosuppressants were given.

Post-transplant management

Immunosuppression was based on tacrolimus and steroids. Target tacrolimus trough levels ranged from 12 to 15 ng/mL for the first two wk, then around 10 ng/mL from two to four wk after LT, and 5–8 ng/mL from one to six months after LT, after which they decreased gradually to < 5 ng/mL more than six months after LT. Intravenous methylprednisolone was administered during the first week after LT, followed by oral prednisolone. Steroids were started during graft reperfusion at a dose of 10 mg/kg, then tapered from 1 to 0.5 mg/kg at one wk after LT, with doses of 0.3 mg/kg from two to four wk after LT, 0.2 mg/kg in the second month, and 0.1 mg/kg in the third month. As a general rule, steroids were withdrawn at the end of the third month. An antiproliferative agent, such as azathioprine or mycophenolate mofetil, was considered as an additional agent, when a patient showed renal dysfunction because of calcineurin inhibitor or had pathological lesions, such as IH or fibrosis. In terms of immunosuppressive regimen for blood type incompatible cases, when a patient is less than one yr old, there is no difference from standard regimen. When a patient is more than one yr old, we check titer of ABO antibody and decrease this < 1:8 with whole blood change transfusion before LT. Additional immunosuppressants were given to inhibit humoral rejection. About 2 mg/kg/day of methylprednisolone and 0.01 μg/kg/minute of prostaglandin E1 were continuously administered through portal vein catheter for one to two wk postoperatively. In addition, 2 mg/kg/day of cyclophosphamide was provided immediately after LT and was switched to 20 mg/kg/day of mycophenolate mofetil from one month after LT (23). Management of immunosuppression therapy was based on clinical information and the results of indirect immunohistochemistry were not informed to clinical staff.

Indication for a liver biopsy was based on the results of laboratory data (liver function tests and fibrosis markers, such as hyaluronic acid, procollagen type III, and collagen type IV), and/or an ultrasound examination. Recipients were evaluated every month at the outpatient clinic after hospital discharge, and blood tests and ultrasound examinations were routinely performed. In terms of autoantibody detection, ANA, ASMA, and LKM-1 antibody were routinely measured every three months (cut off titer of both ANA and ASMA are < 1:40 and cut off level of anti LKM-1 is 17.0 index). IgG quantitation was also included in blood tests every three months conducted at the outpatient clinic.

Histopathologic evaluations

Liver biopsies were taken percutaneously with a 16- or 18-gauge biopsy needle, when clinically indicated, and sections were routinely stained with hematoxylin–eosin. Two well-experienced pathologists evaluated all of the samples, with pathological assessments based on published criteria (2, 15, 24). Fibrosis was evaluated based on METAVIR score (25, 26). In this study, METAVIR score was used for the evaluation of fibrosis. This scoring system was specially designed for patients with hepatitis C and originally designed in the context of post-necrotic cirrhosis. It is useful to exclude inter- and intra-observer variation in the assessment of liver biopsy. IH was defined as periportal or periseptal hepatitis with a predominantly lymphoplasmacytic necroinflammatory infiltrate, with or without lobular involvement and portal-portal or central-portal bridging necrosis, and with the formation of liver cell rosettes and nodular regeneration (23).

Indirect immunohistochemistry technique

Indirect immunohistochemistry assessment was performed with post-transplant serum samples from the 43 recipients and normal human liver tissues from two living liver donors (20, 21). Post-transplant serum samples were collected at one certain point during follow-up period, and liver biopsy was performed before collecting these samples. All serum samples were collected between 1.7 and 184.1 months after LT (median, 50.0 months), and stored at −80 °C. Donor liver tissue was routinely collected at the beginning of living liver donor operations, which is called time 0 biopsy. Histologically, normal liver tissues from one male and one female donor with blood type O were selected to avoid the effect of gender specificity and blood type compatibility between sera and tissue. All liver tissues were fixed in 10% buffered formalin and embedded in paraffin.

A recipient serum sample diluted 1:100 was used for raising the primary antibody. Peroxidase labeled rabbit anti-human IgG antibody (Dako, Glostrup, Denmark) diluted at 1:100 was used as a secondary antibody. Paraffin sections were deparaffinized, then, the antigen was retrieved by microwaving in a 0.01 M citrate buffer (pH 7.0). Sections were incubated in 1% normal rabbit serum for blocking non-specific bindings, after which the primary antibody was added and incubated overnight at 4 °C. After washing three times with phosphate-buffered saline for five min to block endogenous peroxidase activity, specimens were incubated with 0.3% hydrogen peroxidase in methanol for 10 min. After washing, the specimens were incubated with the secondary antibody for one h. Color development was performed using diaminobenzidine solution for 10 min and counter-staining was performed with hematoxylin. As a negative control, a serum sample taken from the same donor was used to raise the primary control antibody.

The evaluation of staining intensity and the number of positive cells were performed as a relative assessment. To avoid being subjective, specimens were randomized and coded before analysis, which was conducted by two independent observers, who evaluated all specimens at least twice within a given interval to minimize intra-observer variation. Any inter-observer differences in scoring were discussed with consensus as the outcome. Both hepatocytes and biliary epithelial cells were evaluated for staining intensity and number of positive cells. According to relative evaluations of the specimens, they were classified as negative, doubtful, positive, and strongly positive, respectively (Figs. 1 and 2). According to our preliminary data, immunohistochemistry between serum samples and liver tissues from the same donor was considered to be negative reaction. Therefore, a reaction between liver tissue and the donor serum sample was established as a negative control. On the other hand, we tried to detect the reaction between the occult antibodies and human liver tissue in this study, which means that it was not sure what types of serum samples or which combination showed positive reactions before this analysis. Consequently, after observing all reactions between two liver tissues and 43 serum samples, we considered the most strongly stained sample as a positive control. When strongly positive reactions were observed in both male and female liver tissues, they were scored as 3. When positive reactions were observed in both or when strongly positive reactions were observed in one and positive reactions were observed in another, they were scored as 2. Weakly positive reactions in both or either were regarded as doubtful, and scored as 1. For analysis of outcome in terms of IH and fibrosis, scores of 0 and 1 were considered to be negative and scores of 2 and 3 positive. As a result, only obviously positive reaction in both types of liver tissues was categorized into the positive group for analysis, otherwise they were categorized into the negative group.

Statistical analysis

IH and fibrosis were set as the endpoint, and disease-free time was computed according to the Kaplan–Meier method. In addition, disease-free time was compared using log-rank analysis. For comparison of the means of quantitative variables, an unpaired *t*-test was used. Qualitative variables were evaluated using cross-tables with a chi-square test. A chi-square test for univariate analysis and logistic regression analysis for multivariate analysis were performed to assess

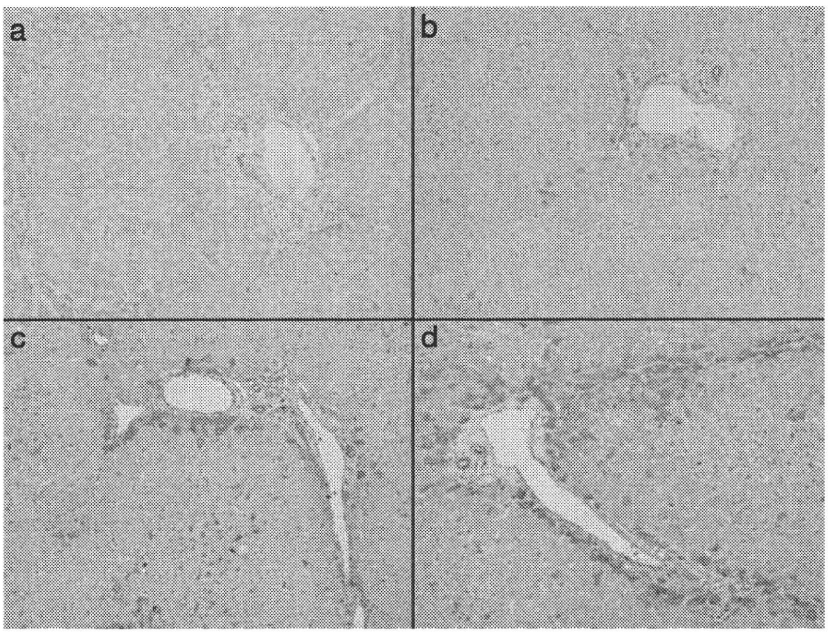

Fig. 1. Classification for staining intensity of indirect immunohistochemistry: Hepatocytes (original amplification ×200). Strongly stained hepatocytes tended to be located in the periportal and/or pericentral area. (a) Negative finding. (b) Doubtful finding (weakly positive reaction). (c) Positive reaction. (d) Strongly positive reaction.

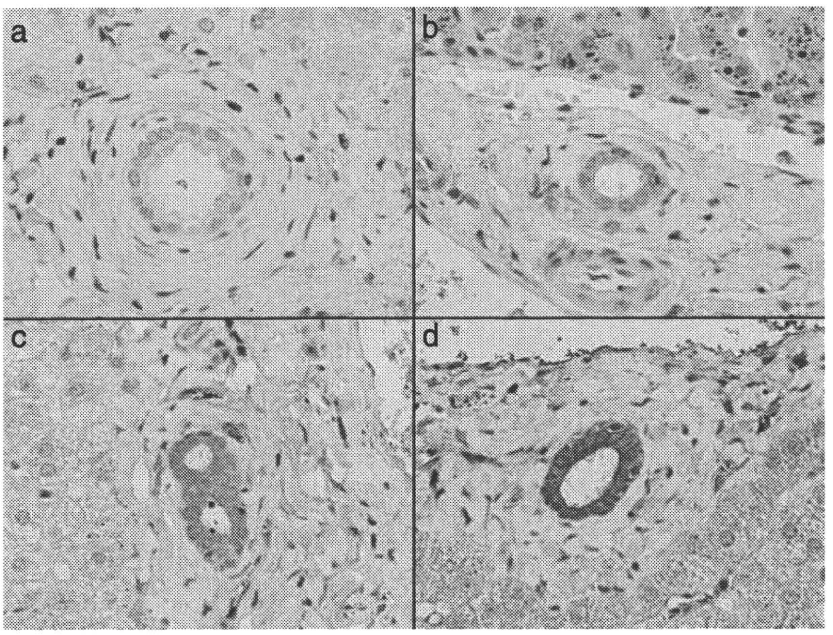

Fig. 2. Classification for staining intensity of indirect immunohistochemistry: Biliary epithelial cells (original amplification ×400). Stained biliary epithelial cells were observed in most of portal areas. (a) Negative finding. (b) Doubtful finding (weakly positive reaction). (c) Positive reaction. (d) Strongly positive reaction.

the risk factors for development of fibrosis and IH. Data are shown as the mean ± standard deviation or median with range. The software package SPSS (version 16.0, SPSS Japan Inc. Tokyo, Japan) was used for statistical analysis, with the level of significance set at $p < 0.05$.

Results

Histological results for post-transplant follow-up biopsy specimens

Fibrosis of grade 1 or more occurred in 13 (30%) of 43 patients. Mean time between LT and initial diagnosis of fibrosis was 4.4 ± 3.6 yr (range, three months to 11.6 yr; median, 5.0 yr). As for the severity of fibrosis, two (5%) cases were F3, four (9%) were F2, and seven (16%) were F1. Two patients classified as F3 showed bridging fibrosis, while in 11 patients classified as F2 and F1, fibrosis was observed in the periportal area. On the other hand, IH was observed in six (14%) of 43 patients. The mean time between LT and initial diagnosis of IH was 6.8 ± 2.7 yr (range 3.7–11.6 yr; median, 6.3 yr). Twenty-one patients (49%) had episodes of late-onset acute cellular rejection, which occurred more than three months after LT.

Results of indirect immunohistochemistry

In terms of evaluation of staining intensity, there was a little discrepancy between observers, but that was subtle and did not influence on the final results of this study. The observers largely concurred on the immunohistochemical findings. Positive hepatocytes were detected in 18 patients, with a score of two given to 12 patients and a score of three to six patients. Strongly stained hepatocytes were generally located in the periportal and/or pericentral area (Fig. 1). Staining pattern of hepatocyte is characterized by diffusely stained cytoplasm. In terms of patient characteristics categorized by indirect immunohistochemistry evaluations, primary diagnosis, recipient gender, recipient age, follow-up period, date of serum sample, and donor age were similar between patients with positive and negative reactions of hepatocytes. In addition, several factors regarding characteristics were shown in Table 2. The antiproliferative agents utilized and steroid therapy tended to be associated with positive reactions for hepatocytes, although those associations were not significant. The presence of autoantibody tended to be higher in the positive group, but not significant. Liver function parameters, including AST, ALT, TB, and GGT, and IgG levels were measured using the same serum samples used for the indirect immunohistochemistry evaluations, and no differences were found between the two groups (data not shown). The occurrence of fibrosis and IH was significantly associated with positive reactions for hepatocytes ($p < 0.001$ and $p = 0.026$, respectively). Furthermore, according to log rank analysis, a positive reactions for hepatocytes were associated with a significantly shorter time to fibrosis (Fig. 3a), as the fibrosis-free period was 6.4 yr (95% CI = 4.3–8.6 yr) in the positive group and 13.8 yr (95% CI = 11.3–16.4 yr) in the negative group ($p = 0.002$). Moreover, a positive reaction for hepatocytes was associated with the occurrence of IH (Fig. 3b). Patients in the positive group had a significantly shorter time to IH, as the disease-free

Table 2. Recipient and donor characteristics based on indirect immunohistochemistry results

	Hepatocytes			Biliary epithelial cells		
	Positive (n = 18)	Negative (n = 25)	p value	Positive (n = 11)	Negative (n = 32)	p value
ABO blood-type compatibility						
Identical	15 (83%)	14 (56%)	0.166	9 (82%)	20 (63%)	0.421
Compatible	2 (11%)	8 (32%)		1 (9%)	9 (28%)	
Incompatible	1 (6%)	3 (12%)		1 (9%)	3 (9%)	
Antiproliferative agent	11 (61%)	9 (36%)	0.103	7 (64%)	13 (41%)	0.187
Azathioprine	7 (39%)	4 (16%)		4 (36%)	7 (22%)	
Mycophenolic acid	4 (22%)	4 (16%)		3 (28%)	5 (16%)	
Mizoribine	0	1 (4%)		0	1 (3%)	
Steroids use						
Ongoing	11 (61%)	8 (32%)	0.058	6 (55%)	13 (41%)	0.423
Withdrawal	7 (39%)	17 (68%)	0.100	5 (45%)	19 (59%)	0.804
History of bolus treatment	15 (83%)	15 (60%)	0.947	8 (73%)	22 (69%)	0.209
Initial withdrawal (days)†	161 ± 150	157 ± 221		112 ± 59	172 ± 216	
R/D gender match						
Mismatch	12 (67%)	11 (44%)	0.142	7 (64%)	16 (50%)	0.434
F to M/M to F	6/6	5/6		6/1	5/11	
No mismatch	6 (33%)	14 (56%)		4 (36%)	16 (50%)	
M to M/F to F	1/5	1/13		1/3	1/15	
Fibrosis (F1/F2/F3)	11 (7/3/1) (61%)	2 (0/1/1) (8%)	**<0.001**	7 (4/2/1) (64%)	6 (3/2/1) (19%)	**0.005**
IH	5 (28%)	1 (4%)	**0.026**	2 (18%)	4 (13%)	0.639
Late-onset ACR*	11 (61%)	10 (40%)	0.172	6 (55%)	15 (47%)	0.661
Presence of autoantibody	8 (44%)	5 (20%)	0.085	6 (55%)	7 (22%)	**0.042**
ANA 1:40	2	3		2	3	
ANA 1:80	1	0		1	0	
ANA 1:2560	1	0		0	1	
ASMA 1:40	2	0		2	0	
LKM	2‡	2§		1¶	3**	
IgG (mg/dL)	1331 ± 425	1346 ± 415	0.911	1337 ± 373	1341 ± 433	0.975

M, male; F, female; LT, liver transplantation; R/D, recipient and donor; M, male; F, female; IH, interface hepatitis; ACR, acute cellular rejection; ANA, anti nuclear antibody; ASMA, anti-smooth muscle antibody; LKM, anti-liver kidney microsomal type 1 antibody; IgG, immunoglobulin G.

*Episode of ACR greater than three months after LT.

†Four patients in the positive group and five in the negative group were treated by steroids throughout the follow-up period.

‡The index levels of anti LKM-1 in two patients were 35.7 and 32.5, respectively (cut off level is 17.0 index).

§The index levels of anti LKM-1 in two patients were 22.5 and 27.5, respectively.

¶The index level of anti LKM-1 in one patient was 35.7.

**The index levels of anti LKM-1 in three patients were 22.5, 27.5, and 32.5, respectively.

period was 8.5 yr (95% CI = 6.0–11.0 yr) in the positive group and 10.3 yr (95% CI = 9.1–11.4 yr) in the negative group (p = 0.048).

On the other hand, positive biliary epithelial cells were detected in 11 patients, of whom a score of 2 was given to 8 and a score of 3 was given to 3 (Fig. 2). In terms of staining pattern of biliary epithelial cells, cytoplasm was diffusely stained. Primary diagnosis, recipient gender, recipient age, follow-up period, date of serum sample, and donor age were similar between patients with positive and negative reactions of hepatocytes. Table 2 also shows patient characteristics categorized by indirect immunohistochemistry evaluations of biliary epithelial cells. The presence of autoantibody was significantly higher in the positive group (p = 0.042). As for liver function tests measured with the same serum samples, there were no differences between the two groups (data not shown). The occurrence of fibrosis was associated with positive reactions for biliary epithelial cells (p = 0.005). According to log rank analysis results, a positive reaction for biliary epithelial cells was associated with a shorter time to fibrosis (Fig. 3c), as the fibrosis-free period was 6.2 yr (95% CI = 3.1–9.3 yr) in the positive group and 11.8 yr (95% CI = 9.3–14.4 yr) in the negative group (p = 0.014). However, this finding was not associated with the occurrence of IH (Fig. 3d), as that disease-free period was 10.2 yr (95% CI = 7.8–13.6 yr) in the positive group and 9.2 yr (95% CI = 7.8–10.6 yr) in the negative group (p = 0.819).

Analysis of risk factors for fibrosis and IH

We investigated the risk factors for development of fibrosis and IH (Table 3). Univariate analysis showed that fibrosis was significantly associated with late-onset acute cellular rejection,

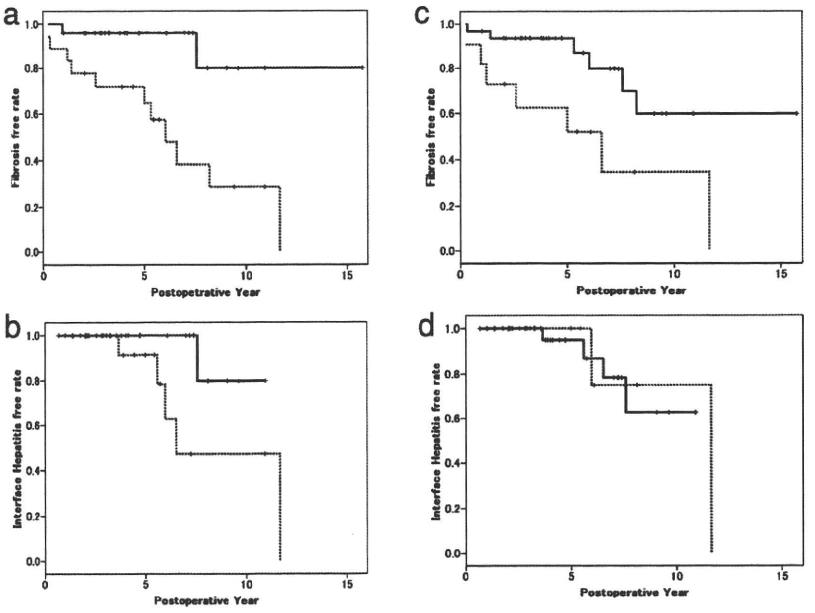

Fig. 3. Comparison of cumulative disease-free rates between the positive and negative group of immunohistochemical assay results. Interrupted and solid lines represent the positive group and the negative group, respectively. (a) Cumulative fibrosis-free rates compared between patients shown positive and negative in indirect immunohistochemistry for hepatocytes. Log rank analysis revealed that a positive reaction for hepatocytes was associated with a significantly shorter time to occurrence of fibrosis ($p = 0.002$). (b) Cumulative IH-free rates compared between patients rated as positive and negative in indirect immunohistochemistry for hepatocytes. A positive reaction was associated with a significantly shorter time to occurrence of IH ($p = 0.048$). (c) Cumulative fibrosis-free rates compared between patients shown positive and negative in indirect immunohistochemistry for biliary epithelial cells. A positive reaction for biliary epithelial cells was associated with a significantly shorter time to occurrence of fibrosis ($p = 0.042$). (d) Cumulative IH-free rates compared between patients rated as positive and negative for biliary epithelial cells. A positive reaction was not associated with occurrence of IH ($p = 0.819$).

development of IH, presence of autoantibodies, and positive reactions for hepatocytes, and biliary epithelial cells. The development of IH was significantly associated with a duration of follow-up more than five yr, donor age < 30 yr old, gender mismatch, episode of late-onset of acute cellular rejection, and positive reactions for hepatocytes. In contrast, the presence of autoantibodies was not associated with the development of IH. In terms of liver function and fibrosis markers, patients with fibrosis showed significantly higher level of procollagen type 3, but other two parameters, hyaluronic acid and collagen type IV, were not significant. ALT was significantly higher in patients with IH. AST also tended to be higher in patients with IH. In addition, primary diagnosis, recipient gender, recipient age, and ABO blood type compatibility were not considered as a risk factor for fibrosis or IH (data not shown).

We entered these variable factors into a logistic regression model for multivariate analysis, which revealed that positive reactions for both hepatocytes and biliary epithelial cells as well as episode of late-onset acute cellular rejection remained independently associated with fibrosis (Table 4). In terms of risk factors for the development of IH, a multivariate analysis could not be performed, because of multicollinearity between the factors (27).

Discussion

The etiology of fibrosis and IH in liver transplant recipients has not been clarified (2, 4, 9, 28–30). In particular, it is not known whether the etiology of fibrosis in pediatric liver recipients is immunological. In terms of the pathogenesis of fibrosis and IH, we hypothesized that recipients might have some antibodies to the transplanted liver that may lead to chronic hepatitis, such as IH, and finally cause fibrosis. Our results suggest that the etiology of IH and fibrosis may be immunological and may be associated with the presence of occult antibodies in recipient's sera. These antibodies reacting to the human liver may be present even before LT or may be produced as a reaction to immune-mediated aggression of transplanted liver. Actually, it remains controversial whether antibodies reactions that were detected in this study cause IH and/or fibrosis. However, there is still a possibility that some antibodies in recipient's sera can react to transplanted liver, which causes IH and/or fibrosis.

Table 3. Risk factors for fibrosis and interface hepatitis

	Fibrosis			Interface hepatitis		
	Positive (n = 13)	Negative (n = 30)	p value†	Positive (n = 6)	Negative (n = 37)	p value†
Follow-up period (yr)						
≤ 5	6 (46%)	17 (57%)	0.526	0	23 (62%)	**0.005**
>5	7 (54%)	13 (43%)		6 (100%)	14 (38%)	
Donor age (yr)						
≤ 30	7 (54%)	7 (23%)	0.050	5 (83%)	9 (24%)	**0.004**
>30	6 (46%)	23 (77%)		1 (17%)	28 (76%)	
R/D gender mismatch	3 (23%)	12 (33%)	0.876	6 (100%)	17 (46%)	**0.014**
Late-onset ACR*	11 (85%)	10 (33%)	**0.002**	6 (100%)	15 (41%)	**0.007**
IH	5 (38%)	1 (3%)	**0.002**	–	–	–
Fibrosis	–	–	–	5 (83%)	8 (22%)	**0.002**
Presence of autoantibodies	7 (54%)	6 (20%)	**0.026**	3 (50%)	10 (27%)	0.256
Indirect immunohistochemistry						
Positive reaction for hepatocytes	11 (85%)	7 (19%)	**<0.001**	5 (83%)	13 (35%)	**0.026**
Positive reaction for biliary epithelial cells	7 (54%)	4 (13%)	**0.005**	2 (33%)	9 (24%)	0.639
Fibrosis marker						
Hyaluronic acid (ng/mL)	46.9 ± 30.3	36.3 ± 28.9	0.217	51.8 ± 50.1	35.9 ± 25.1	0.225
Procollagen type III (units/mL)	1.73 ± 0.94	1.18 ± 0.33	**0.006**	1.25 ± 0.38	1.36 ± 0.66	0.577
Collagen type IV (ng/mL)	217.1 ± 114.1	162.9 ± 78.4	0.137	209.3 ± 145.1	174.4 ± 83.3	0.588
Liver function						
AST (units/L)	33.5 ± 20.2	29.1 ± 9.1	0.469	39.3 ± 29.4	29.0 ± 8.8	0.081
ALT (units/L)	24.5 ± 23.0	20.5 ± 11.5	0.560	37.2 ± 31.0	19.2 ± 10.4	**0.008**
GGT (units/L)	30.0 ± 22.6	34.0 ± 66.6	0.771	22.0 ± 13.4	34.5 ± 60.8	0.278
TB (mg/dL)	0.72 ± 0.27	0.93 ± 0.86	0.241	0.82 ± 0.37	0.88 ± 0.78	0.769
IgG (mg/dL)	1401 ± 437	1313 ± 409	0.545	1671 ± 510	1286 ± 378	0.128

R/D, recipient and donor; LT, liver transplantation; ACR, acute cellular rejection; IH, interface hepatitis; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; TB, total bilirubin.
*Episode of ACR greater than three months after LT.
†Chi-square test in univariate analysis.

Table 4. Factors related to development of fibrosis

	Multivariate p value†	Odds ratio	95% CI
Late-onset ACR*	**0.034**	36.3	1.3–1013.6
IH	0.295	6.1	0.2–177.1
Presence of autoantibodies	0.652	1.7	0.2–16.1
Indirect immunohistochemistry			
Positive reaction for hepatocytes	**0.020**	22.1	1.6–302.3
Positive reaction for biliary epithelial cells	**0.047**	20.0	1.0–385.1

ACR, acute cellular rejection; IH, interface hepatitis; CI, confidence interval.
*Episode of ACR greater than three months after LT.
†Logistic regression model in multivariate analysis.

These antibodies probably react to normal human liver tissue as well. As a result, positive reactions can be observed with this indirect immunohistochemistry technique.

In the present study, the development of fibrosis was associated with positive reactions for both hepatocytes and biliary epithelial cells, whereas IH was associated with positive results for hepatocytes but not biliary epithelial cells. It is assumed that fibrosis may be provoked by a variety of antibodies that target hepatocytes as well as biliary epithelial cells. On the other hand, IH may be caused by antibodies that mainly target hepatocytes. These results also indicate that fibrosis is a consequence of a variety of types of immunological morbidity.

The theoretical basis of this indirect immunohistochemistry technique is similar to that of the anti-LKM antibody identification method (31). A previously reported immunohistochemical staining method that utilized paraffin-embedded tissue samples was able to identify specific immunoglobulins reactions without non-specific immunoglobulin staining (32, 33). With this method, the reaction between unknown antibodies in patient serum to the normal liver tissue element could be demonstrated. It is difficult to predict what types of serum samples would show positive reactions before the analysis. Therefore, the most strongly stained specimen was established as positive control in this analysis. On the other hand, although antigen–antibody reactions can be detected with this technique, the subcellular location of the potential autoantigen is still unclear with this technique. This indirect immunohistochemistry technique we used in this study is unable to specify the kinds of antibodies involved and the nature of antigens. A method for detection of the kind of occult antibodies and

the nature of the targeted antigens in liver tissue is considered to be the next step. Moreover, an examination of post-transplant liver allograft biopsy for antibody staining could be helpful to understand the etiology of IH and fibrosis.

It was reported that the presence of autoantibodies in liver transplant recipients is associated with the presence of chronic hepatitis, which leads to chronic hepatitis and progressive fibrosis (3, 7, 10, 12, 13). However, the presence of autoantibodies was not an independent factor in our analysis. Moreover, late-onset acute cellular rejection was shown to be a risk factor for both fibrosis and IH, whereas the presence of autoantibodies was a risk factor only for fibrosis. It is assumed that the presence of autoantibodies is one of the clinical signs that indicate immunologically activated status, and late-onset acute cellular rejection may occur as a consequence of an immunologically activated status. These results suggest that it is difficult to predict immunological activation by monitoring the presence of known autoantibodies and that unknown antibodies may play an important role in the development of fibrosis and IH, as well as late-onset acute cellular rejection.

This study has certain limitations. To confirm the mechanism of the production of occult antibodies by hepatocytes and biliary epithelial cells, another approach will be needed. In our series, the biopsies were taken as clinically indicated and the serum samples were taken shortly after this; therefore, the timepoint was variable. Indirect immunohistochemistry with longitudinally collected serum samples may be useful to indicate when these antibodies are produced. According to preliminary data of longitudinal investigations in one patient who showed positive reaction for hepatocytes in this present study, the result was negative with pretransplant serum sample, and it turned out to be positive with a sample collected on postoperative day 7 and strongly positive with a sample collected on postoperative day 28 in terms of hepatocyte staining. This patient had episodes of late-onset acute cellular rejection, and then showed fibrosis two yr after LT. These results indicate that antibodies to liver tissue may be produced after LT. However, more detailed examination will be needed to clarify this issue.

In conclusion, indirect immunohistochemistry between liver recipient serum between human liver tissue showed antigen–antibody reaction on hepatocytes or biliary epithelial cells, which indicates that there is a possibility that the pathogenesis of fibrosis and/or IH observed in pediatric liver transplant recipients in the chronic phase is immunological. To improve the long-term outcome of liver transplant recipients, an investigation into the mechanisms of late-onset immunological morbidity is crucial. The present results suggest that immune-mediated reactions by occult antibodies underlie the pathogenesis of fibrosis and IH observed in liver transplant recipients.

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Fluorescent In Situ Hybridization 1p/19q Deletion/Imbalance Analysis of Low-Grade and Atypical Meningiomas

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Abstract

The chromosomal 1p/19q state was analyzed in 16 low-grade meningiomas and 7 atypical meningiomas using fluorescent in situ hybridization (FISH) analysis. Chromosome 1p aberrations were observed in all atypical meningiomas, but in only one low-grade meningioma. Atypical meningiomas showed 19q deletion or imbalance, suggesting chromosomal instability of 19q. A small group of low-grade meningioma showed 19q aberrations. FISH 1p/19q deletion/imbalance analysis is a sensitive method for detecting chromosome aberrations of meningiomas and provides useful information for grading of meningiomas. Patients with low-grade meningioma with chromosomal instability of 1p/19q should be followed up carefully. Assessment of the chromosomal state by FISH might be of crucial importance in the clinical management of meningiomas.

Key words: meningioma, fluorescent in situ hybridization, chromosome 1p/19q, chromosomal instability

Introduction

Meningiomas are common intracranial tumors arising from arachnoidal cells. Most meningiomas are benign (World Health Organization [WHO] grade I), but atypical meningiomas (WHO grade II) constitute between 4.7% and 7.2% of meningiomas, and anaplastic meningiomas (WHO grade III) account for 1.0–2.8%.[11] Both atypical and anaplastic meningiomas demonstrate histological and clinical features suggesting aggressive potential, and show a tendency for tumor recurrence, even after complete resection. Histological findings, such as increased cellularity, uninterrupted patternless growth, and mitotic figures may be helpful, but these subtle differences may not be readily apparent on routine hematoxylin-eosin staining, and may require measurement of MIB-1 labeling index to assess the proliferative activity. High MIB-1 labeling index is considered to be closely correlated with the histological grading of malignancy. The WHO classification of atypical meningioma requires increased mitotic activity of 4 or more mitoses per 10 high-power fields.[10] However, the correct cutoff value for these continuous variables is difficult to establish. Histological grades and MIB-1 labeling index have a fairly good correlation, but MIB-1 labeling index shows significant overlap between benign, atypical, and anaplastic meningiomas.[11] Therefore, these diagnostic markers of proliferation have limitations, and there is a need for a more precise, efficacious, and objective approach to the diagnosis and grading of meningiomas. Genetic characterization is another possible alternative for grading of meningiomas.[2,16,18–20]

The present study explored the potential diagnostic utility of genetic alterations by comparing the histological features and chromosomal states in a series of meningiomas using fluorescent in situ hybridization (FISH).

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Table 1 Cases of low-grade and atypical meningiomas

Case No.	Age (yrs)/ Sex	Location	Subtype	WHO grade	MIB-1 LI (%)	FISH diagnosis		1p Normal (%)	1p Abnormality (total, %)	19q Normal (%)	19q Abnormality (total, %)
						FISH 1p	FISH 19q				
1	42/M	olfactory groove	trans	I	0.2	normal	normal	96.2	3.8	92.6	7.4
2	62/M	jugular foramen	men	I	1.5	normal	normal	97.3	2.7	97.7	2.3
3	69/F	suprasellar	men	I	0.6	normal	normal	90.1	9.9	92.6	7.4
4	71/F	parasagittal	trans	I	0.8	normal	mono.	89.9	10.1	44.5	55.5
5	69/F	convexity	fibrous	I	0.1	normal	normal	92.1	7.9	90.0	10.0
6	72/F	convexity	angio	I	0.1	normal	normal	98.5	1.5	92.0	8.0
7	70/F	parasagittal	fibrous	I	0.4	normal	normal	94.5	5.5	95.5	4.5
8	75/M	falx	angio	I	0.7	normal	poly.	75.5	24.5	55.0	45.0
9	80/F	tuberculum sellae	men	I	1.2	normal	normal	96.5	3.5	99.0	1.0
10	70/M	tentorium	men	I	2.5	normal	normal	94.5	5.5	99.0	1.0
11	44/M	parasagittal	men	I	1.0	normal	normal	96.0	4.0	95.5	5.0
12	54/F	petroclival	men	I	0.8	normal	normal	92.6	7.4	94.4	5.6
13	41/M	convexity	men	I	2.0	normal	normal	100.0	0	96.0	4.0
14	45/F	parasagittal	fibrous	I	0.4	normal	normal	94.7	5.3	85.0	15.0
15	64/F	convexity	metapla	I	0	normal	normal	87.0	13.0	99.0	1.0
16	22/F	convexity	trans	I	7.8	def. deletion	normal	15.0	85.0	95.0	5.0
17	56/F	tentorium	atyp	II	8.2	dispro. deletion	amp.	3.6	96.4	9.4	90.6
18	48/F	ventricle	atyp	II	5.0	deletion with imb.	amp.	8.5	91.5	8.7	91.3
19	62/F	convexity	atyp	II	3.5	dispro. deletion	normal	7.8	92.2	63.8	36.2
20	78/M	convexity	atyp	II	6.3	def. deletion	def. deletion	15.0	84.5	16.7	83.3
21	79/F	convexity	atyp	II	3.5	def. deletion	imb.	17.3	82.7	44.2	55.8
22	55/F	tentorium	atyp	II	1.5	def. deletion	normal	14.0	86.0	63.0	37.0
23	60/F	sphenoid ridge	atyp	II	1.2	def. deletion	normal	24.1	75.9	57.5	42.5

Contd.

Materials and Methods

Tumor samples were collected from 16 consecutive surgically resected low-grade meningiomas, and 7 consecutive atypical meningiomas including 3 archival and 4 fresh samples obtained from 23 patients, 16 females and 7 males aged 22–80 years (mean 60.3 years). The locations of the tumors are shown in Table 1. All tumors were classified by histological examination and graded according to WHO guidelines.[10] The diagnostic criteria for atypical meningioma were based on increased mitotic activity defined as 4 or more mitoses per 10 high-power fields or 3 or more of increased cellularity, small cell with high nucleus/cytoplasm ratio, prominent nucleoli, uninterrupted patternless or sheet-like growth, and necrotic foci.[10]

FISH analysis used a touch preparation of fresh samples. The material was prepared with Carnoy's fixation and denatured at 80°C for 5 minutes. Cell copy numbers were investigated by FISH using Vysis® LSI 1p36/1q25 and LSI 19q13/19p13 Dual-Color Probe (Abbott Laboratories, Abbott Park, Ill., U.S.A.). Signals were counted for each hybridization in randomly chosen regions of at least 200 nonoverlapping nuclei with intact morphology. Parallel touch preparation methods, prepared from the same surface as the hybridized slide, were used to confirm the successful collection of tumor cells. Parallel touch preparations for each tumor was stained with Papanicolaou stain and reviewed by a cytopathologist to identify the fraction of tumor cells. Tissues from archival samples were prepared as described before.[12]

Assessment and interpretation of FISH results used the previously published criteria modified for

Table 1, contd.

Subtype of 1p abnormality (%)								Subtype of 19q abnormality (%)							
		Instability except for mono. and poly.								Instability except for mono. and poly.					
Mono.	Poly.	Def. deletion	Dispro. deletion	Imb.	Amp.	Other aberration	Total	Mono.	Poly.	Def. deletion	Dispro. deletion	Imb.	Amp.	Other aberration	Total
0	0	1.9	0	0	1.9	0	3.8	0.6	2.5	2.8	0	0	1.9	0	4.7
0	2.7	0	0	0	0	0	0	0	2.3	0	0	0	0	0	0
0	0.9	9.0	0	0	0	0	9.0	0.5	0.9	6.0	0	0	0	0	6.0
0	1.6	3.2	0	1.1	4.2	0	8.5	48.9	0	1.8	0	0	4.8	0	6.6
0.4	4.8	0.4	0	1.5	0.8	0	2.7	0.9	4.4	0.9	0	0	3.8	0	4.7
0.5	0	0.5	0	0	0.5	0	1.0	0	7.5	0.5	0	0	0	0	0.5
0	5.5	0	0	0	0	0	0	0.5	3.5	0	0	0.5	0	0	0.5
3.0	12.5	8.5	0	0.5	0	0	9.0	1.5	37.5	0.5	0	2.5	3.0	0	6.0
0	3.5	0	0	0	0	0	0	0	1.0	0	0	0	0	0	0
0	0.5	5.0	0	0	0	0	5.0	0	0.5	0.5	0	0	0	0	0.5
0	4.0	0	0	0	0	0	0	0	5.0	0	0	0	0	0	0
0	6.5	0.9	0	0	0	0	0.9	0	3.7	1.9	0	0	0	0	0
0	0	0	0	0	0	0	0	0	4.0	0	0	0	0	0	0
0	5.3	0	0	0	0	0	0	2.4	10.8	0	1.8	0	0	0	1.8
5.0	7.0	1.0	0	0	0	0	1.0	0	0	1.0	0	0	0	0	1.0
0	0	83.0	0.0	2.0	0	0	85.0	0	2.0	1.0	0	2.0	0	0	3.0
6.8	0	37.4	44.2	4.9	0.4	2.7	89.6	10.8	1.0	1.5	0	0	74.8	2.5	78.8
2.3	3.8	23.0	32.0	27.2	3.2	0	85.4	1.3	5.7	0.9	0	0.9	82.7	0	84.5
9.1	1.4	28.7	44.8	4.8	1.7	1.7	81.7	9.5	1.0	9.0	1.4	0.5	14.8	0	25.7
0	0	84.5	0	0	0	0	84.5	0	0.3	82.1	0	0	0.9	0	83.0
0.6	0.6	76.3	0	5.2	0	0	81.5	0	0	3.4	0	52.4	0	0	55.8
11.0	0	60.0	0	15.0	0	0	75.0	2.0	19.0	4.0	0.0	4.0	8.0	0	14.0
0.9	0	73.1	0	1.9	0	0	75.0	0	16.4	15.7	0.7	1.5	8.2	0	26.1

Abnormalities exceeding 20% are underlined. angio: angiomatous meningioma, amp: amplification, atyp: atypical meningioma, def.: definitive, dispro.: disproportional, fibrous: fibrous meningioma, FISH: fluorescent in situ hybridization, imb.: imbalance, LI: labeling index, men: meningothelial meningioma, metapla: metaplastic meningioma, mono.: monosomy, poly.: polysomy, trans: transitional meningioma, WHO: World Health Organization.

brain tumors (Table 2).[12] Deletion or imbalance was defined as alterations detected in >20% of the nuclei. Comparison of intact nuclei from fresh cell preparations and truncated nuclei in paraffin sections showed that nuclear truncation resulted in approximately 5–9% signal loss.[1,4] Nuclear truncation due to sectioning or other artifacts in the hybridization process could increase the false-positive signal deletion. Therefore, we adopted a cut-off value of 20% for FISH analysis.[12]

All tumors were evaluated for the expression of proliferation-associated nuclear antigen Ki-67 (MIB-1) using formalin-fixed paraffin sections. Images captured with a digital camera were analyzed quantitatively with a free software package, Scion Image (Scion Corporation, Frederick, Md., U.S.A.), to count the number of cells labeled with MIB-1. All results were reviewed by manual measurements to confirm this computer-assisted technique.

The relationships between 1p/19q deletion or imbalance and histological grade were analyzed using the Fisher exact test. P values of <0.05 were considered statistically significant. All samples were tested according to an institutional review board-approved protocol.

Results

The diagnoses and histological subtypes of the tumors are shown in Table 1. Table 1 also summa-

Table 2 Definitions of chromosome status by fluorescence in situ hybridization (FISH)

FISH—Chromosome 1p36
Deletion: one or less signal for chromosome 1p36 (ratio 0/1, 0/2, 1/2, 1/3, 1/4 etc.)
Definitive deletion: one or less signal for chromosome 1p36 in relation to normal reference probe signals on chromosome 1q25 (ratio 1/2, 0/2)
Disproportional deletion: one or less signal for chromosome 1p36 with disproportion of the ratio of the reference probe (ratio 1/3, 1/4 etc.)
Deletion with reference deletion: chromosome 1p36 deletion with deletion of reference region (ratio 0/1)
Imbalance: imbalance of the ratio of the reference probe to chromosome 1p36 with more than two reference probe signals (ratio 2/3, 3/4, 3/5 etc.)
Polysomy: simultaneous increase of both signals (ratio 3/3, 4/4, 5/5 etc.)
Monosomy: simultaneous decrease of both signals (ratio 1/1)
Amplification: target signal > reference signal per cell (ratio 3/2 etc.)
Normal: no deletion, no imbalance and no polysomy detected by FISH with the probes used (ratio 2/2)

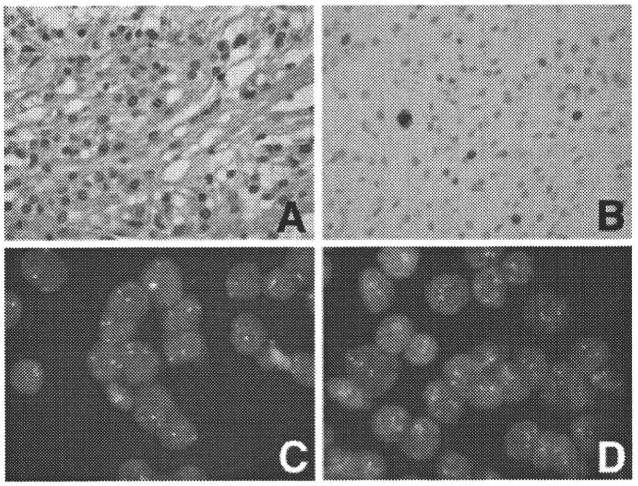

Fig. 1 A, B: Photomicrographs of samples of atypical meningioma showing uninterrupted patternless growth with mitotic figures (A: hematoxylin and eosin stain, original magnification ×400), and MIB-1 labeling showing active proliferation (B: immunohistochemistry for Ki-67, original magnification ×400). C, D: Fluorescent in situ hybridization analysis of chromosomal arms 1p/19q showing 1p36 deletion status with a 2:1 ratio of control (*green*) signals and target (*red*) signals (C), and 19q13 imbalance status with 3:2 and 5:2 ratios of control (*green*) signals and target (*red*) signals (D).

rizes the genomic abnormalities of 1p/19q status detected by FISH (Fig. 1) in the 23 tumors studied. The mean MIB-1 labeling index was not significantly higher for atypical meningiomas (mean 4.2%) than for low-grade meningiomas (mean 1.3%). All 7 atypical meningiomas showed chromosome 1p deletion including disproportional deletion and imbalance, and the cell population with 1p deletion accounted for over 50% of the tumor in all cases. In contrast, 1p deletion was detected in only one of the 16 low-grade meningiomas. Six of the 7 atypical meningiomas had 19q instability including deletion, imbalance, or amplification, whereas 2 of 16 low-grade meningiomas demonstrated 19q monosomy or polysomy (Table 1). These FISH analyses showed the genomic abnormalities of 1p/19q status of atypical meningiomas were significantly different from those of low-grade meningioma ($p < 0.05$ for both locations).

Discussion

FISH is a powerful tool for the assessment of target deoxyribonucleic acid dosages in interphase nuclei without paired blood samples and has been used for evaluating numerical and structural genomic aberrations in various tumors.[5,12,13,17] The primary advantages are applicability to archival, formalin-fixed paraffin-embedded material and morphological preservation, which is particularly attractive for studies on heterogeneous tissue samples without the need for microdissection. Detectable genomic alterations include deletion, imbalance, polysomy, translocation, and gene amplification. However, consensus criteria for assessment and interpretation of analytical results in brain tumors have not been established.[12] The present study used the criteria modified for brain tumors (Table 2). According to these criteria, "deletion" is defined as only one or less target signal (1/2, 1/3, 1/4 etc.) and subdivided into "definitive" deletion (1/2, 0/2), "disproportional" deletion (1/3, 1/4 etc.), and deletion "with reference deletion" (0/1). "Imbalance" is defined as disproportion of the signal ratio (2/3, 3/4, 3/5 etc.). FISH is a sensitive method for evaluating numerical genomic abnormalities at the cytogenetic level. It is also important to distinguish "disproportional" deletion and imbalance from "definitive" deletion among these chromosomal instabilities.

The present study used FISH with chromosome-specific probes to evaluate 1p and 19q chromosome aberrations in low-grade and atypical meningiomas. Our results showed genomic abnormalities in the 1p/19q status of atypical meningiomas, which were significantly different from the absence of abnormalities in low-grade meningioma. A few low-grade meningiomas showed abnormality of 1p/19q. Genetic changes associated with chromosome instability on chromosome 1p are important in both tumorigen-

esis and tumor progression in meningiomas. Frequency of loss of heterozygosity (LOH) on chromosome 1p tends to increase with tumor grade, as LOH on 1p was observed in 40% of grade 2 meningiomas and 70% of grade 3 meningiomas.[16] Chromosome 22q deletion and/or mutation of neurofibromatosis type 2 gene (*NF2*) are most frequently detected as the earliest genetic aberrations and found in meningiomas of all malignancy grades, indicating that inactivation of this gene represents an early event in the pathogenesis of meningiomas.[14,15,19,20] LOH for loci on chromosome 22q and mutations in the *NF2* locus probably do not play a role in the development of malignancy in meningiomas.[16] Thus, the assessment of chromosome 22q is not suitable for the alterations associated with progression of meningiomas. LOH associated with meningioma progression has been extensively researched,[2,16,18] but only limited data on chromosomal aberrations by FISH are available.[3,6,13-15]

FISH analysis should be assessed as numerical genomic abnormalities at the cytogenetic level, not as the rate of chromosome gain or loss.[6,14,15] Assessment of FISH deletion status is useful for the evaluation of chromosomal instability of meningiomas. The frequency of deletions is proportional to histological grade and chromosome 1p deletion was identified in 23% of low-grade and 56% of atypical meningiomas.[3] Deletion of 1p occurred in 23–35% of low-grade meningiomas, and in 56–92% of the atypical meningiomas.[3,13] However, these previous FISH studies did not discriminate between deletion and imbalance status.

The present study showed that FISH 1p/19q deletion/imbalance analysis is a sensitive method for detecting chromosome aberrations of meningiomas and provides useful information for the grading of meningiomas. Specific details relative to FISH deletion status were not provided in all previous reports, so the frequency of specific deletion types cannot be compared with the present results. However, our results indicate that the frequency of 1p deletion in low-grade meningiomas may be lower than previously reported and further investigation is needed. The present study also demonstrated that atypical meningiomas have chromosomal instability of 19q including deletion, imbalance, and amplification.

Previous FISH studies analyzing 1p status in brain tumors have not compared MIB-1 labeling index and the chromosomal state.[3,6,13-15] The present results showed that MIB-1 labeling index is useful for grading meningiomas, but there is significant overlap. In contrast, FISH analysis of the 1p/19q status clearly distinguished atypical meningioma from low-grade meningioma. The diagnosis of meningioma grade may depend on both assessment of proliferative state using MIB-1 labeling index and chromosomal state using FISH in the future.

In general, genetic changes precede morphological changes. Our results showed that a small group of low-grade meningiomas have 1p/19q aberrations. Whether such chromosome instability is an indicator of biological aggressiveness in histologically benign subgroups of meningiomas is extremely interesting. Some authors reported that meningiomas with chromosome 1p loss show the higher rate of recurrence occurring independently from the histological classification.[7-9] In our series, some low-grade meningiomas had FISH 1p definitive deletion and 19q monosomy or polysomy. Further investigation is needed to determine whether these low-grade meningiomas with chromosomal abnormality have high rates of recurrence and progression. Characterization of those genetic aberrations might facilitate the early identification of tumors with high intrinsic risk of progression. We plan to follow up patients with histologically benign meningiomas with a high percentage of 1p/19q deletions or imbalance.

FISH 1p/19q deletion/imbalance analysis is a sensitive method for detecting chromosome aberrations in meningiomas and provides useful information for the grading of meningiomas. Assessment of both chromosomal state by FISH and proliferative state by MIB-1 labeling index might be important in the clinical management of meningiomas.

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Commentary

Meningiomas are the second most common primary tumor of the central nervous system. Atypical (grade II) and anaplastic/malignant (grade III) meningiomas, which are associated with a higher rate of recurrence and aggressive growth, account for about 10% of all meningiomas. To find certain sensible and reliable indicators for grading of meningioma is crucial and valuable. The genetic aspects of meningiomas have been studied intensively during the past decade. Chromosome 22q, 1p, 9q, 14q and 10q aberration have been detected to correlate with meningioma progression. In the present article, the authors conclude that FISH 1p/19q deletion/imbalance analysis is a sensitive method for detecting chromosome aberrations of meningiomas and provides useful information for the grading of meningioma. Chromosome 1p/19q loss of heterozygosity has been regarded as a molecular genetic characteristic for oligodendroglioma. Can 1p/19q deletion/imbalance be an indicator for meningioma grading, treatment choice and prognosis expectation? The authors have made a valuable contribution. To answer this question finally, more larger studies are expected in the future and the 1p/19q status in anaplastic meningioma needs further investigation.

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Identification of *TINF2* gene mutations in adult Japanese patients with acquired bone marrow failure syndromes

Mutations of the genes involved in human bone-marrow failure syndromes (BMFS) have been identified in components of the telomerase- and telomere-associated genes, including the *TINF2* gene on chromosome 14q11·2, which encodes the 40 kDa TIN2 component of the telomere-associated shelterin protein complex (Calado & Young, 2008; Savage *et al*, 2008; Walne *et al*, 2008; Walne & Dokal, 2009). Clinically, it is very important to identify patients with pathogenic mutations in the telomere- or telomerase-associated genes, because these patients will probably exhibit refractoriness to conventional immunosuppressive therapy (IST) (Calado & Young, 2008). Several recent studies showed heterozygous *TINF2* mutation in 1–5% of patients with acquired aplastic anaemia (AA) (Walne *et al*, 2008; Du *et al*, 2009). The subjects of these studies were Caucasian, Black and Hispanic. Analysis of the *TINF2* gene among adult Asian populations of AA and myelodysplastic syndrome (MDS), to the best of our knowledge, had never been done. The largest controlled epidemiological study reported that the incidence of AA in the West was 2cases/million/year, but was about two- to three-fold higher in Asia (Issaragrisil *et al*, 2006). Therefore, we carried out an investigation to determine whether mutations in *TINF2* could be found in our cohort of adult Japanese patients with acquired BMFS, and if so, at what frequency. We screened exon 6 of *TINF2*, as it was previously found to be a potential hotspot for disease-associated mutations (Walne *et al*, 2008; Du *et al*, 2009), among 142 Japanese patients who were diagnosed with acquired AA or MDS refractory anaemia between 1993 and 2006 at the Nippon Medical School Hospital. We excluded AA and MDS patients who were found to carry mutations in the telomerase *TERC* or *TERT* gene. We identified two AA patients (1·4%) with *TINF2* heterozygous mutations, which were P283H and n865-866 di-nucleotide CC deletion (Fig 1A). The n865-866 di-nucleotide CC deletion in the *TINF2* gene is a novel mutation that has not been previously identified. These mutations were not found in 300 healthy controls. Because of the lack of biological sample from the relatives of the patients as well as other tissues of the patients, it was not possible to determine whether these were segregational or germline mutations. Using Southern blotting technique, we compared the length of telomeres of mononuclear cells in AA patients who carried the *TINF2* mutations to those of healthy age-matched controls. As shown in Fig 1B, AA patients with *TINF2* mutations (Patients 1 and 2) showed much shorter telomere lengths than those of healthy age-matched controls.

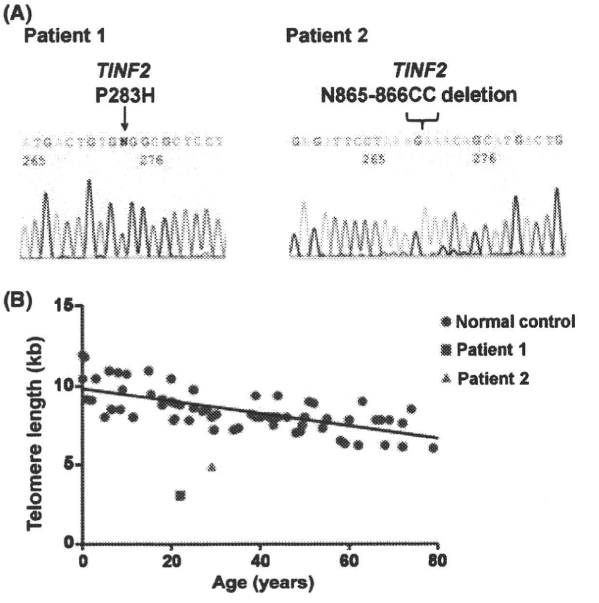

Fig 1. Identification of *TINF2* mutations and telomere length measurements. (A) Gene mutations identified by direct sequencing. In the case of the n865-866 di-nucleotide CC deletion (Patient 2), the *TINF2* PCR product was sub-cloned into pCR2·1-TOPO expression vector and sequenced. (B) telomere lengths of patients with *TINF2* mutations. Patient 1: 3·1 kb, Patient 2: 4·9 kb.

The clinical characteristics of these two patients with *TINF2* mutation are shown in Table I. Both of the patients with *TINF2* mutations were diagnosed with severe AA with no physical features of Dyskeratosis Congenita or its severe variant Hoyeraal-Hreidarsson syndrome (HH) (Walne *et al*, 2008) and showed no clinical response to IST. We attempted treatment of our patients with *TINF2* mutations with metenolone, which is a dihydrotestosterone (DHT)-based anabolic steroid with androgenic properties, but they did not show any favourable clinical responses, unlike a previous report of favourable haematological response in BMFS patients with *TERT* mutations upon androgen treatment (Calado *et al*, 2009). In summary, we report here for the first time *TINF2* natural mutations in 2/142 Japanese patients with acquired BMFS, which is at about the same frequency (1·4%) as reported in patients of other ethnic groups (Caucasian, Black and Hispanic) (Walne *et al*, 2008; Du *et al*, 2009).

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Table I. Clinical characteristics of patients with *TINF2* gene mutations.

Patient	Gene	Location of mutation	Age (years)	Sex	Diagnosis	Family history	Physical anomaly	Neutrophils ($\times 10^9$/l)	Hb (g/l)	Reticulocytes ($\times 10^9$/l)	Platelets ($\times 10^9$/l)	Chromosome abnormality	Shortened telomere	Treatment
1	*TINF2*	P283H	22	M	sAA	–	–	0·4	76	16·8	19	–	+	No response to IST
2	*TINF2*	Del n865-866	29	M	sAA	–	–	0·35	69	15·0	22	–	+	No response to IST

sAA, severe aplastic anaemia; IST, immunosuppressive therapy.

Authorship and disclosures

HY was the principal investigator and takes primary responsibility for the paper. HY, KI, JT, HT and KD recruited the patients. HY, KI, YM and FK performed the laboratory work for this study. HY, HL and KD analyzed the data and wrote the paper. The authors reported no potential conflicts of interest.

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