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Telomere shortening and karyotypic alterations in hepatocytes in long-term transplanted human liver allografts

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Conflicts of Interest
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Summary

The long-term fate of aged liver allografts in young recipients who received grafts from older donors is unknown. We evaluated graft aging by analyzing hepatocytic telomere length and karyotypic changes. Seventeen pediatric individuals who underwent living-donor liver transplantation for congenital biliary diseases were selected. At a median of 10.4 years post-transplant, ten had tolerated grafts with weaned off immunosuppressants, and seven had idiopathic post-transplantation hepatitis. Fluorescence *in situ* hybridization was used to evaluate the telomere signal intensity (TI) and karyotypic changes. First, we measured predictive age-dependent TI decline with regression analysis of donor livers. The mean TI at the earliest (within a year) and latest biopsies was significantly lower than the predicted TI of the studied allografts. With univariate analysis, a higher abnormal karyotype ratio in the donor liver was correlated with development of idiopathic post-transplantation hepatitis. With multivariate analysis that included clinical parameters, a greater TI decline at the earliest biopsy was correlated with the development of idiopathic post-transplantation hepatitis. In conclusion, graft aging as measured by TI decline and donor abnormal karyotype ratio was associated with idiopathic post-transplantation hepatitis of long-term transplanted liver allografts.

Introduction

Maintenance of long-term graft function is important in liver transplant patients, in particular pediatric patients, who are expected to have a longer post-transplant life than adult patients [1–5]. Pediatric patients often receive grafts from their parents with living-donor liver transplantation. Livers from older donors have worse graft survival rates in human liver transplantation [6,7], and therefore, accurate evaluation of graft aging and senescence is expected to provide critical data for therapeutic intervention in long-term grafts. In renal transplantation, biological organ age, as determined by markers of cellular senescence such as $p16^{INK4a}$, is reported to be a prognostic indicator, followed by donor age [8,9]. Although a recent report has shown rejuvenation of aged grafts as assessed with immunohistochemistry for the senescence marker protein-30 in the setting of human adult-to-pediatric liver transplantation [10], it is unknown what role cellular senescence plays in determining the lifespan of liver allografts in young recipients who receive grafts from older donors.

Many insults including rejection can contribute to post-transplant damage [11]. Late post-transplant biopsies

frequently show chronic hepatitis of unknown cause (idiopathic post-transplant hepatitis, IPTH), and this condition can cause late graft dysfunction leading to cirrhosis [11–13]. Tolerance has been reported more frequently in liver transplant recipients than after transplantation of other organs. Graft senescence in idiopathic post-transplant hepatitis (IPTH) and tolerated grafts (TG) after long-term transplantation is unknown. In the present study, we focused on chromosomal changes including telomere shortening [14,15] and abnormal karyotypic changes in liver allografts as indicators of senescence.

Telomere length in chronic hepatitis or cirrhosis is significantly lower than that in normal liver of the same age [16]. Sustained cellular turnover in chronic liver disease accelerates cellular senescence or a crisis as a result of telomere shortening [17–21]. We compared the relative telomere length in grafts over a similar follow-up period using quantitative fluorescence *in situ* hybridization (Q-FISH). In addition, we analyzed the karyotypic changes in hepatocytes and the presence of recipient-derived hepatocytes in sex-mismatched liver grafts. Hepatic polyploidy increases after partial hepatectomy and in aged animals [22]. A proportion of tetraploid hepatocytes is normally present in the liver, and the ploidy of hepatocytes tends to increase with aging [22,23]. Recently, polyploid hepatocytes have been shown to have more extensive proliferative potential [24,25].

Materials and methods

Case selection

Between 1990 and August 2008, 735 pediatric patients underwent living-donor liver transplantation from their parents in Kyoto University Hospital. We used the registered database to select patients who satisfied the following conditions: minimum of eight years after transplantation, 18 years old or younger at the time of transplant, history of congenital biliary diseases, available pre-implantation liver tissue (donor liver), and two or more post-transplant biopsies including one within a year after transplantation (Figure 1). Two hundred eighty-one patients were followed for more than 8 years after liver transplantation, and 137 patients underwent the late biopsies during the study period from August 2006 to August 2008.

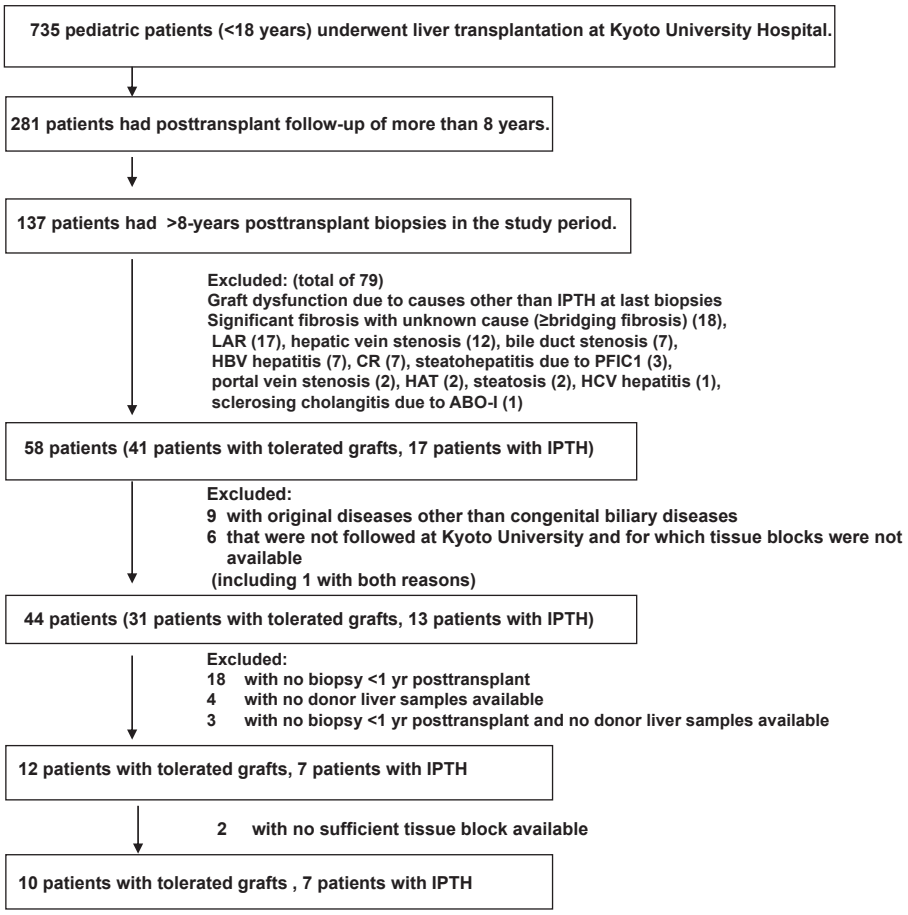

Figure 1 Patient selection flow diagram. IPTH, idiopathic post-transplantation hepatitis; LAR, late onset acute rejection; HBV, hepatitis B virus; CR, chronic rejection; PFIC1, progressive familial intrahepatic cholestasis type 1; HAT, hepatic artery thrombosis; HCV, hepatitis C virus; ABO-I; ABO blood type-incompatible liver transplantation.

Definition of TG and IPTH

We selected two groups of patients: those with tolerated graft (TG) and those with idiopathic post-transplant hepatitis (IPTH). TG was defined as a condition in which the allograft functioned normally and lacked histological signs of rejection in the absence of immunosuppression or a low maintenance dose of tacrolimus at the time of the last biopsy [26–28]. TG patients were selected from those who had participated in our immunosuppression withdrawal protocol [28].

In the IPTH group, biopsies showed histology of chronic hepatitis with interface activity that cannot be ascribed to any specific cause, and diagnosis of IPTH was made. IPTH has been called de novo autoimmune hepatitis (AIH) by some groups. This condition is commonly associated with positive autoantibodies, such as anti-nuclear antibody and elevation of IgG, and biochemically and histopathologically resembles AIH in non-transplanted patients [11,12]. Increasing evidence suggests that late acute rejection, de novo AIH, and IPTH are part of an overlapping spectrum of immune-mediated late allograft damage in long-term post-transplant patients [13].

The details regarding patient selection are shown in Figure 1. A total of 41 patients in the TG group (30%) and 17 patients with IPTH group (13%) were selected at the time of the late biopsy. After excluding patients with original disease other than congenital biliary diseases and patients with no tissue available, 10 patients in the TG group and 7 in the IPTH group were included in the study.

Informed consent that was approved by the Ethics Committee of the Faculty of Medicine, Kyoto University, was obtained from patients or their parents at the time of biopsy.

Laboratory data and histological analysis

Histological analysis was performed on tissue samples that had been fixed in formalin and embedded in paraffin. All specimens were interpreted by four pathologists (AM-H, SS, TT, and HH).

The biopsies within a year of the transplant were taken based on a clinical suspicion of rejection. For subsequent biopsies, protocol biopsies were performed at approximately 5 and 10 years after transplantation in patients who had participated in our immunosuppression withdrawal protocol. For the remaining patients, liver biopsies were performed when clinically indicated.

The severity of necroinflammation was graded according to the METAVIR scoring system [29]. Postoperative laboratory data logged at the time of the last biopsies in TG were used, together with data taken at the time of observation of IPTH (Table 1).

Measurement of telomere signal intensity (TI) with Q-FISH

The mean TI was assessed at time zero (donor liver) and at several times after transplantation (including >10.4 years). As reference data, regression analysis of TI was performed using normal donor livers of the study population ($n = 17$). The median age of the 17 donors was 32 years (range, 26–41 years), of which five were male and 12 were female. No donors histologically showed steatosis, fibrosis, necrosis, or inflammation.

A total of 34 liver specimens were available for TG and 24 for IPTH for Q-FISH analysis. There was an average of 3.4 specimens per patient in TG, and 3.4 in IPTH. Q-FISH was performed using a PNA probe that was specific for $(TTAGGG)_n$ sequences, (K5325, DakoCytomation, Denmark). After pre-treatment according to the manufacturer's instructions, the slides were hybridized overnight at room temperature. After washing, tyramide signal amplification was used. Then, anti-FITC/HRP (AbD Serotec, Oxford, UK; 1:200) was applied for 1 hour, followed by biotinylated tyramide (CSA system kit, DakoCytomation) for 10 min, and then Alexa Fluor 568-conjugated streptavidin (Molecular Probes, Eugene, OR; 1:400) for 2 hours at room temperature. The slides were mounted in DAPI (1:2000, Dojindo Laboratories, Tokyo, Japan).

Fluorescence signals were visualized under an All-in-One Fluorescence Microscope (BZ-8000 Biozero, Keyence, Osaka, Japan). Blue (DAPI) and red (Alexa) signals were captured at 600 × magnification. To compare absolute fluorescence intensities between different images, the integration times used for the individual color channels were saved automatically for each image [30]. All exposure times were held constant. Pictures were taken at 25 ms for both the Alexa Fluor 568 images and the DAPI images. Deconvoluted fluorescence images were used to identify telomere spots within nuclei.

Image analysis

The TI value was measured on the images using Meta Series Software 7.5.4 (Molecular Devices, Downingtown, PA) and was expressed in relative fluorescence units (RFU). For each slide, the mean TI of hepatocyte nuclei in 10 fields (600 ×) was calculated.

In situ hybridization for X and Y chromosomes

After pre-treatment, FISH was performed on pretreated slides for the X chromosome (CEP X, spectrum green) and Y chromosome (CEP Y, spectrum red) (Abbott Molecular Inc. Des Plaines, IL). The slides were denatured for 10 min at 73 °C and hybridized overnight at 37 °C

Table 1. Clinical and histological findings of the study population.

No.	Recipient's gender/age (yr)	Donor's gender/age (yr)	Period post-LT (yr)	METAVIR score of late specimens	Laboratory findings at late biopsy		
					AST (IU/l)	ALT (IU/l)	T-Bil (mg/dl)
Tolerated graft group							
1	F/1	F/27	14.7	A0F0	20	18	0.5
2	F/1	F/26	12.2	A0F0	33	38	0.9
3	F/1	M/29	12.3	A0F0	27	22	1.2
4	F/0	F/28	8.8	A0F0	36	27	0.4
5	F/1	F/32	10.6	A0F0	26	18	1.5
6	M/4	M/29	8.5	A0F0	31	26	0.7
7	F/3	F/37	9.8	A0F0	35	18	0.8
8	F/0	F/29	9.6	A0F0	22	20	1.1
9	F/1	F/32	8.8	A0F0	30	20	1.1
10	M/0	F/32	8.4	A0F0	25	18	1
Median	1	29	9.7		29	20	1.0
Idiopathic post-transplant hepatitis group							
11	F/4	M/36	17.4	A1F2	60	40	1.1
12	F/1	F/34	14.2	A1F3	35	30	1.2
13	M/2	M/34	11.4	A1F3	46	60	0.9
14	F/9	M/40	11.4	A1F4	300	718	16.2
15	F/6	F/32	10.0	A2F2	184	109	3.9
16	M/6	F/34	10.4	A2F3	137	128	0.9
17	F/18	F/41	8.4	A2F3	121	114	0.8
Median	6	34	11.4		121	109	1.1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LT, liver transplantation; No., number; SD, standard deviation; T-Bil, total bilirubin, yr, year.

Fibrosis stage (METAVIR): F0 (absent); F1 (portal fibrosis), F2 (bridging fibrosis), F3 (precirrhosis), F4 (cirrhosis).

Necroinflammation grade (METAVIR): A0 (absent), A1 (mild), A2 (moderate), A3 (severe). Normal ranges in Kyoto University Hospital for ALT level, 13–33 IU/l; for AST level, 8–42 IU/l; for T-Bil level, 0.3–1.3 mg/dl.

and were analyzed at a magnification of 600 ×. Female and male donor livers were stained as controls.

A proportion of tetraploid hepatocytes is normally present in the liver, and thus, the karyotypes expected in normal males are XY and XXYY and in normal females are XX and XXXX. The number of hepatocyte nuclei was determined by counting the total number in 10 fields (600 ×). Hepatocytes with a single chromosome signal (X only in female grafts and X or Y only in male grafts) comprised hepatocytes with a sectioning artifact; it was not known whether they were diploid or aneuploid, so we excluded them from further analysis.

The hepatocyte chimerism percentage was calculated by counting the total number of chimeric hepatocytes in 10 foci for each slide in sex-mismatched cases; in particular, hepatocytes with a Y chromosome signal in female-to-male liver transplantation were considered to be hepatocyte chimeras.

Statistical analysis

Comparisons between data were made using the Student's *t*-test after confirmation with the F-test and Shapiro-Wilk W-test that the parameter obeyed normal and equal distributions. For analysis of the abnormal karyotype ratio, positive square root transformation of the parametric values was done, and an unpaired Student's *t*-test was performed after confirmation that the parameter obeyed normal distribution with equal variances. For analysis of the clinical background, a *t*-test or U-test was performed. Regression analysis was used to test for relationships between quantitative variables. Cox proportional hazard analysis was performed to examine the relationships between several factors and occurrence of IPTH. A P-value < 0.05 was considered significant. For statistical analysis, JMP Start Statistics version 5 was used (Statistical Discovery Software SAS Institute, Cary, NC).

Results

Clinical profiles of long-term transplanted grafts

The first group, the TG group, was composed of 10 patients who exhibited normal liver function tests (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) after a long-term follow-up period (range, 8.4–14.7 years; median, 9.7 years) (Table 1). The original diseases in these patients were all congenital biliary diseases, including nine cases of biliary atresia and one case

of congenital biliary dilatation. Two of them received left or lateral grafts from their father, and eight received grafts from their mother. Patients with TG took either a low maintenance dose of tacrolimus monotherapy (trough level <1.5 ng/ml), or they had discontinued tacrolimus at the time of the late protocol biopsies. There were minimal histological abnormalities, such as slight perivenular fibrosis and non-specific mild inflammatory changes. The grade and fibrosis score using the METAVIR scoring system was A0 F0 in all cases (Figure 2a, Table 1). No vascular or biliary complications were detected in any TG patients.

The second group, the IPTH group, was composed of seven patients with IPTH. The original disease in all seven cases was congenital biliary atresia. The patients survived the initial grafts but exhibited abnormal liver function tests after a similar follow-up period (range, 8.4–17.4 years; median, 11.4 years). Three of the IPTH patients received left or lateral grafts from their father, and four received grafts from their mother. A triple immunosuppression regimen of tacrolimus, prednisolone, and mycophenolate mofetil was used in all of these patients. Histology showed plasma-cell-rich interface hepatitis with lymphoid cell spillover into the lobules (activity score A1∼A2) and progressive portal fibrosis (F4 in one case, F3 in four cases, and F2 in two cases at the time of diagnosis; see Figure 2b, Table 1). In summary, the histology of the IPTH group showed chronic hepatitis, with mild to moderate activity, and progressive fibrosis, whereas histologically, there was no score (A0 F0) in the TG group.

There were no significant differences in clinical data including follow-up duration, original disease, and age between patients in the two groups. We observed significant differences in the clinical data between TG and IPTH patients (AST, $P = 0.007$; ALT, $P = 0.001$) (Table 1). The median times from transplantation to earliest biopsies were 47 days (range, 12–104 days) in the TG group ($n = 10$) and 31 days (range, 14–264 days) in the IPTH group ($n = 7$) ($P = 0.27$). The histological diagnoses of the earliest biopsies were acute cellular rejection (ACR) in five cases, nonspecific mild lobular inflammation in three cases, and mild steatosis in two cases in TG. The diagnosis in all seven IPTH cases was ACR.

Hepatocellular telomere signal intensity (TI) decline in long-term transplanted grafts

The mean TI of the liver allograft was assessed at time zero (donor liver) and at several times after transplantation, including the earliest biopsies within 1 year of the transplant ($n = 17$) and the late biopsies at a median of 10.4 years post-transplant ($n = 17$).

A photomicrograph of FISH is shown in Figure 3a. To evaluate the aging, the TI of the hepatocytes was first assessed in biopsied liver samples of the 17 normal donors using Q-FISH. Regression analysis revealed an age-dependent TI decline (Figure 3b). In subsequent analysis (below), this regression line was used as a reference for the TI analysis in the transplants. TI in donor livers was not significantly different between TG and IPTH ($P = 0.66$). Figure 3b shows data for individual patients. TI was significantly lower in the allografts than the predicted TI for each allograft age.

The predicted TI of each allograft at the time of earliest and last biopsies was calculated using the TI of each donor liver and the annual rate of telomere shortening (−0.11) of the reference line. As a result, the TI declined significantly relative to the predicted TI of the allograft in both groups at each time period ($P < 0.05$) (Figure 3c).

The TI decline soon after transplantation was calculated from the difference between the TI of the donor liver and the TI at the earliest biopsies, and the TI decline of each patient late post-transplant was calculated from the difference between the TI of the donor liver and the TI at the last biopsy. The TI declines in the early post-operative period and in the long-term post-operative period (mean ± SD) were 3.96 ± 3.50 in TG and 2.83 ± 2.55

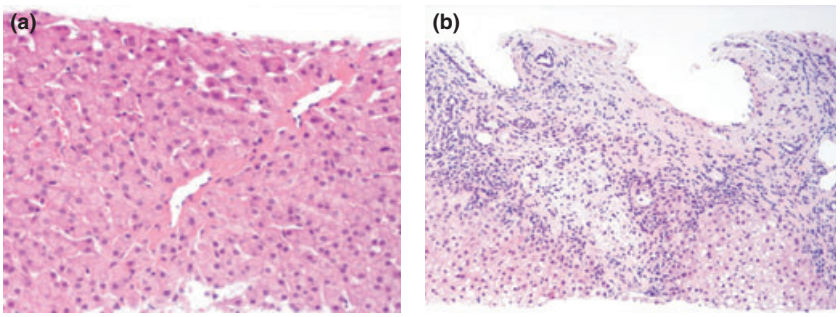

Figure 2 Histology of long-term transplanted allografts. (a) Liver allograft biopsy of a patient with a stable graft (TG) 8.5 years after liver transplantation (H&E staining, original magnification, 200×). (b) Liver allograft biopsy of a patient with a idiopathic post-transplant hepatitis (IPTH) 11.4 years after transplantation showing spillage of lymphoid cells into the lobules and progressive portal fibrosis (H&E 100×).

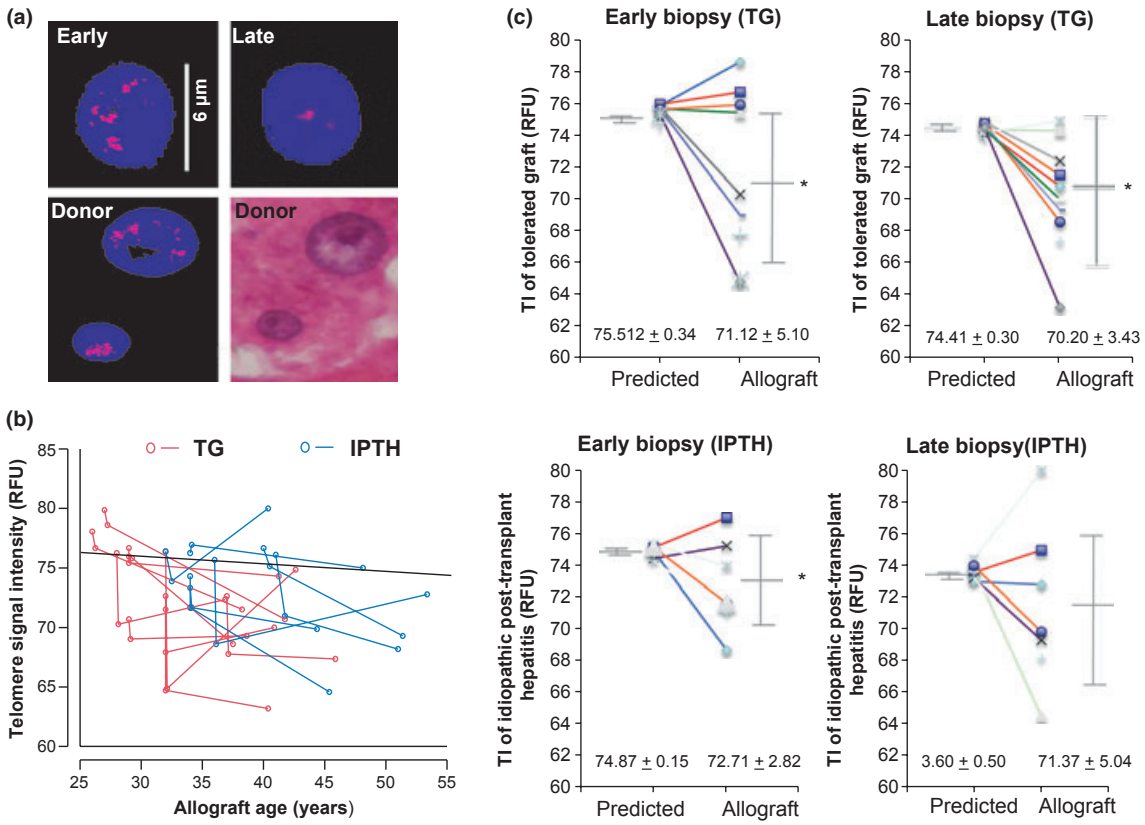

Figure 3 Hepatocyte-specific telomere shortening in patients. (a) Telomere PNA FISH. The image shows telomere-specific FISH analysis of the specimen. Hepatocyte nuclei were stained blue (DAPI; mean diameter, 6 μm), and the telomeres were stained purple (original magnification, 400×). To confirm that these nuclei are hepatocyte nuclei, H&E staining was performed on the same specimen as telomere FISH. (b) Data for the sequential mean TI of hepatocytes in 10 fields in TG (red lines) and IPTH (blue lines). Each individual line in the graph represents data for an individual patient. Regression analysis of donor livers revealed an age-dependent TI decline ($Y = 78.80 - 0.11X$, $R^2 = 0.038$, $P < 0.0001$), which is shown by the black line (RFU, relative fluorescence units). The horizontal axis corresponds to the age of the allograft. (c) The allograft TI (the mean ± SD) was lower than the predicted TI (the mean ± SD) of each allograft calculated from the regression line of the donor livers at the early post-transplant period (<1 year) and at the long-term post-operative period in TG and IPTH. Each line represents one patient. $*P = 0.024$ for allograft TI vs. predicted TI for early biopsies in TG (upper left), $*P = 0.045$ for allograft TI vs. predicted TI for early biopsies in IPTH (lower left), $*P = 0.0037$ for allograft TI vs. predicted TI at last biopsies in TG (upper right), and $P = 0.14$ for allograft TI vs. predicted TI for last biopsies in IPTH (lower right).

in IPTH at the earliest biopsies, and 4.87 ± 2.90 in TG and 4.17 ± 4.58 in IPTH at the last biopsies. The TI declines in the early post-operative period and in the long-term post-operative period were not significantly different between the TG group and IPTH group ($P = 0.46$, $P = 0.73$, respectively).

XY FISH analysis of long-term transplanted allografts

To study karyotypic changes and to determine their relationship with the last histological status of the graft (Figure 4a–d), a FISH assay with X- and Y-chromosome paints was performed in the last biopsies of our 17 patients at a median of 10.4 years post-transplant and in their donor grafts ($n = 10$, TG; $n = 7$, IPTH) (Table 2).

One TG female patient and two IPTH female patients received transplants from male donors. A large majority of the hepatocytes in these grafts had an X-Y chromosomal configuration (Table 2). In TG, 0.42% (1/238 hepatocytes) of hepatocytes were X-X-configured female diploid hepatocytes, whereas the percentages in the two IPTH cases were 0.8% (1/132 cells) and 4.55% (7/154 cells) (Figure 4a, upper panel). Of the two male patients who received transplants from female donors, one was in the TG group and one was in the IPTH group. The chromosomal configuration of most hepatocytes was XX, indicating their donor origin. We observed that 3.4% (6/175 hepatocytes) of hepatocytes in TG and 4.1% (5/121) of hepatocytes in IPTH were recipient-derived hepatocytes (XY-configured and Y-positive) (Figure 4a, lower panel).

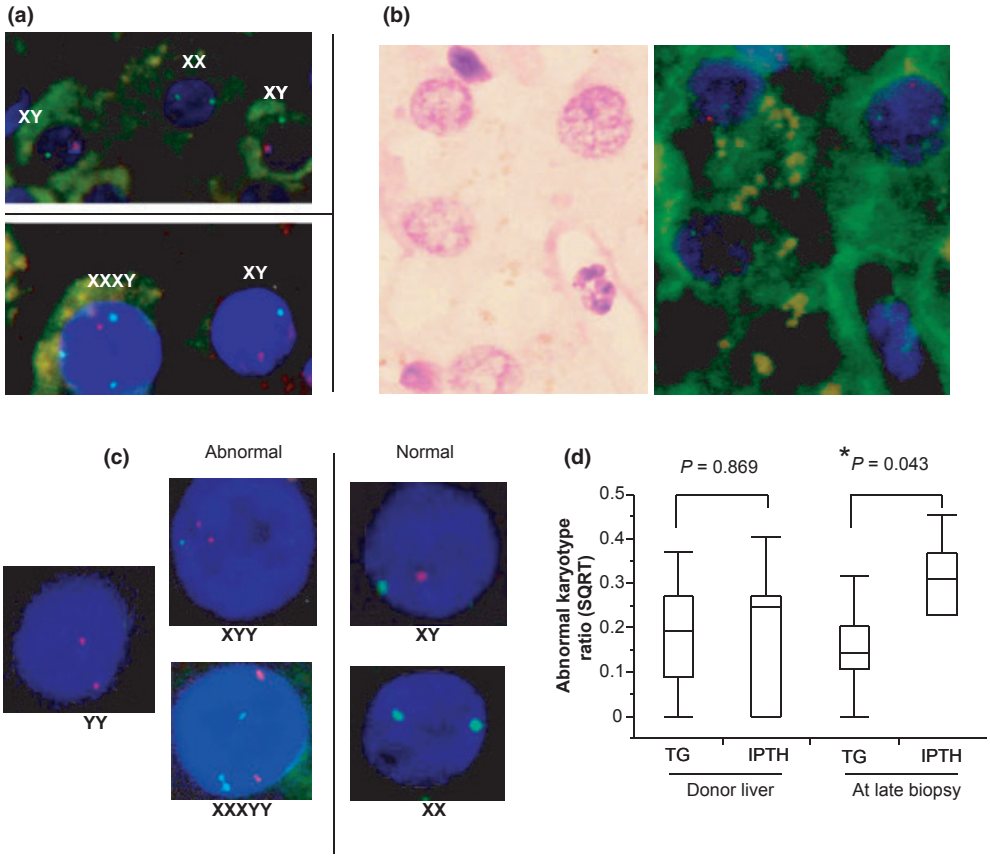

Figure 4 FISH analysis of X- and Y-chromosomal signals. (a) The upper photograph shows a typical case of male-to-female liver transplantation. Two hepatocellular nuclei (stained with DAPI) are visible, consisting of X (stained green)-chromosomal signals and Y (stained red)-chromosomal signals, which indicate the donor origin, and a hepatocyte with XX chromosomes, suggesting a hepatocyte of the recipient (original magnification, 600×). The lower photograph shows a typical FISH displaying XXXY in a female-to-male transplantation (original magnification, 600×), suggesting an aneuploid hepatocyte of recipient origin. (b) To confirm that these nuclei are hepatocyte nuclei, we performed H&E staining on the same specimen as XY FISH. H&E staining of the hepatocytes (left panel) and X, Y FISH (right panel) are shown on the same specimen. The X chromosome is stained green, and the Y chromosome is stained red. (Original magnification of H&E staining, 400×; FISH, 600×). (c) Three typical hepatocytes with abnormal karyotype signals. Normal diploid cell signals are shown on the right (original magnification, 600×). (d) Abnormal karyotype ratio (SQRT, square root) in donor livers and at the late biopsies in TG (n = 10) and IPTH (n = 7). *P = 0.043 for abnormal karyotype ratio in TG vs. IPTH for late biopsies.

The proportion of Y-chromosome-positive recipient-derived hepatocytes (hepatocyte chimerism) was therefore low in both the TG and IPTH groups. To exclude the possibility that blood cells may have been scored accidentally, hepatocytes were identified with HE staining of the same specimen as used for FISH (Figure 4b).

We also observed a variety of abnormal karyotypic changes in hepatocytes that were not consequences of normal nuclear division in the donor liver, including YY, XYYY, XXXY, and XXYY (in female donors), and odd numbers of X and Y signals, such as YYY, XYY, XXY, XXX, XYYYY, XXXYY, and XXXXX configurations (Figure 4c). These complex karyotypic changes were probably due to hepatocyte fusion and/or unequal cell division. In donor livers, the degree of abnormal karyotype was not significantly different in TG (median, 3.74%) and IPTH (median, 6.02%) (P = 0.869). After long-term transplantation, a median of 9.62% of hepatocyte nuclei in IPTH had abnormal karyotypes; in TG, the percentage was 2.02%. Hepatocytes with abnormal karyotypes were more common in IPTH than TG at the late biopsies (P = 0.043) (Figure 4d).

Univariate and multivariate Cox proportional hazard analysis

To assess the factors that influenced the development of IPTH, univariate and multivariate Cox proportional

Table 2. Karyotypic changes in hepatocytes of the allografts.

No.	D-R	Abnormal karyotypes (No. of nuclei)					(A)	(B)	(A)/(B) (%)
Tolerated graft group									
1	F-F	XXX (2)					2	166	1.20
2	F-F	XXX (2)					2	123	1.63
3	M-F	YY (1)	XXY (1)	XYY (3)			5	207	2.42
4	F-F	XXX (2)					2	203	0.99
5	F-F	XXX (3)					3	203	1.48
6	M-M	XXY (2)	XYY (5)				7	70	10.0
7	F-F	XXX (2)					2	83	2.40
8	F-F	XXX (2)					2	72	2.80
9	F-F	–					0	38	0.00
10	F-M	XXX (6)	XXXXX (1)				7	71	9.90
Idiopathic post-transplant hepatitis group									
11	M-F	YY (1)	XXY (6)	XXX (3)			10	104	9.62
12	F-F	–					0	65	0.00
13	M-M	XYY (3)	XXXY (1)				4	39	10.3
14	M-F	YY (1)	XXY (5)	XYY (1)	XXXY (1)	XXXYY (1)	9	66	13.6
15	F-F	XXX (6)	XXXXX (1)				7	34	20.6
16	F-M	XXX (1)	XXY (1)				2	38	5.30
17	F-F	XXX (4)					4	44	9.10

(A), total numbers of nuclei with abnormal karyotypes; (B), total numbers of nuclei with normal karyotypes; D-R, donor-recipient; F, female; M, male; No., number; X, X chromosome; Y, Y chromosome; Normal male hepatocytes are XY or XXYY; normal female hepatocytes are XX or XXXX.

Table 3. Univariate analysis of the correlation between clinicopathological parameters and occurrence of IPTH in transplanted patients.

		95% confidence interval		
Variables	HR	Lower	Upper	*P*-value
Recipient age (≥4 yr versus <4 yr)	7.31	−2.473309	−0.075822	0.0325*
Donor age (≥33 yr versus <33 yr)	4.11	−2.185947	0.2146591	0.1436
ACR (presence or absence)	1.78E-7	−1549.712	1534.1707	0.0507
TI decline in the first year (≥6 versus <6)	2.02E-07	−1745.92	1761.3345	0.1004
Abnormal karyotype ratio (SQRT) in the donor liver (≥0.2449 versus <0.2449)	11.4	−2.727856	−0.17531	0.0220*
GRWR (≥1.4% versus <1.4%)	7.78E-02	0.3129467	2.7669985	0.0085*

ACR, acute cellular rejection; GRWR, graft/recipient weight ratio; HR, hazard ratio; SQRT, square root; TI, telomere signal intensity.
*Significant ($P < 0.05$).

hazard analyses were performed using continuous variables including recipient age, donor age, abnormal karyotype ratio in the donor livers, TI decline in the first year (the difference between TI in the donor livers and TI at the earliest biopsies), the presence or absence of ACR within a year, and graft-recipient weight ratio (GRWR).

With univariate analysis of these parameters, patients with a higher abnormal karyotype ratio in the donor livers (square root of the ratio ≥0.2449) had a greater risk of occurrence of IPTH than patients with a lower abnormal karyotype ratio in the donor livers (<0.2449) ($P = 0.022$) (Table 3). In addition, patients ≥4 years of age and with GRWR <1.4% had a greater risk of occurrence of IPTH than patients <4 years old and with a GRWR ≥1.4% (Table 3).

Multivariate analysis showed that grafts exhibiting ≥6 TI decline within a year had a greater risk of IPTH than those exhibiting a TI decline less than 6. In addition, patients who were ≥4 years old at the time of transplantation and who experienced ACR within a year had a greater risk of IPTH than those who were <4 years and who experienced no episode of ACR (Table 4).

Discussion

We observed a greater telomere reduction than that predicted by chronological age and a higher proportion of abnormal karyotypes in hepatocytes in long-term transplanted liver allografts, suggesting that allografts age more rapidly than the normal population when transplanted. Even in TG, the telomere was shorter than expected for

Table 4. Multivariate analysis of the correlation between clinicopathological parameters and occurrence of IPTH in patients after pediatric living-donor liver transplantation.

Variables	HR	95% confidence interval		*P*-value
		Lower	Upper	
Recipient age (≥4 yr versus <4 yr)	4.78E-17	−11249.5	11287.1	0.0486*
Donor age (≥33 yr versus <33 yr)	4.89E-9	−7577.60	7596.70	0.0931
ACR (presence or absence)	3.69E-17	−13576.9	13614.7	0.0328*
TI decline in the first year (≥6 versus <6)	2.44E-25	−17734.9	17791.6	0.0190*
Abnormal karyotype ratio (SQRT) in the donor liver (≥0.2449 versus <0.2449)	3.57E-9	−12543.0	12562.5	0.1179
GRWR (≥1.4% versus <1.4%)	1.41	−1.80180	1.45522	0.8096

ACR, acute cellular rejection; GRWR, graft/recipient weight ratio; HR, hazard ratio; SQRT, square root; TI, telomere signal intensity.
*Significant ($P < 0.05$).

the graft age. Accelerated TI decline in hepatocytes within a year of transplantation may be a general process in all grafts. This decline is probably due to premature aging following the acute stress of transplantation and the high rate of cell turnover that occurs during graft regeneration immediately after transplantation [23].

We initially hypothesized that IPTH may show more progressive telomere shortening than TG due to high cell turnover [17,19]. A study showed that cellular senescence in the explanted livers of young children is associated with damage rather than corresponding to an age-dependent phenomenon [31]. However, we observed no significant difference in telomere reduction between TG and IPTH at the late biopsy. Telomere shortening does not necessarily reflect the long-term graft status in TG and IPTH, which differ clinically and histologically. The telomere length in hepatocytes was already shortened during the early post-transplant period. According to our multivariate analysis, IPTH patients had more ACR episodes than those in the TG group. ACR may contribute to further early TI decline in hepatocytes. IPTH shows the strongest correlation with previous ACR episodes [12,13,32]. ACR includes immune-mediated injury directed towards bile ducts or the vascular endothelium, rather than hepatocytes [13]. IPTH is thought to be an alloimmune response against hepatocytes [12,13,32]. An increase in senescent cells in association with telomere shortening has been confirmed in the mouse model of ischemia-reperfusion injury [33]. Therefore, hepatocyte damage related to ischemia-reperfusion injury, rather than ACR, is likely to be a major factor in the accelerated TI decline in the early post-transplant period. On the other hand, from the viewpoint of telomere shortening in the early post-transplantation phase, ≥6 TI decline was a risk factor for late dysfunction of the graft. This finding is clinically significant in the examination of follow-up of high-risk allografts.

Other parameters, such as recipient age and donor age, are also related to the outcome of pediatric liver transplantation [6,7]. Development of fibrosis post-transplant is related to factors such as the duration of cold ischemia, age at time of transplantation, high age/donor-recipient ratio, and partial graft [5]. In addition to these factors, we found a higher proportion of hepatocytic abnormal karyotypes in IPTH compared to TG at the last biopsies, suggesting that IPTH progresses to hepatocyte aging more rapidly than TG.

Whether aneuploidy is the result of chronic liver damage or causes chronic damage remains unknown. Unexpectedly, we found that donor livers had hepatocytes with abnormal karyotypes. The donor livers had no steatosis, fibrosis, or inflammation. Polyploid hepatocytes are found in all mammalian species, but the functional significance of this conserved phenomenon remains unknown [24]. In humans, such polyploid hepatocytes may be frequently observed, and additional studies are required. Recent studies have shown that hepatocytes that have undergone extensive proliferation are highly aneuploid [24,25]. We hypothesize that hepatocytes may undergo more extensive proliferation in IPTH patients than in TG patients. It was interesting that grafts with increased abnormal karyotypic changes from the start showed a greater chance of developing IPTH, regardless of donor or recipient age.

We must also emphasize the limitations of the study. The study was retrospective in design. In addition, reproducible quantitation to measure the hepatocyte telomere length and abnormal karyotypic changes in paraffin-embedded liver tissue was technically difficult, even though thin slices may minimize the influence of background autofluorescence [34]. Additionally, patients in the IPTH group received grafts from older donors than those in the TG group, and therefore, grafts in IPTH patients were likely to have undergone a greater telomere reduction and to have had greater numbers of hepatocytes with abnormal karyotypes. The number of patients was not large, and thus, it is still too early to draw conclusions about factors associated with the development of IPTH.

In conclusion, our data confirmed accelerated telomere shortening relative to the graft age after transplantation, probably reflecting high cell turnover and oxidative stress. More hepatocytes with abnormal karyotypes were seen in dysfunctional grafts compared with stable grafts. Determining abnormal donor karyotypic changes and telomere decline in the early post-transplant period in correlation with the donor age should provide useful information about the likelihood of later dysfunction.

Authorship

WA: performed study, collected data, and analyzed data. AMH: designed and performed study, collected data, and wrote the paper. TT: analyzed data and contributed important reagents. SH: performed study. SS: collected data. KT, SU and HH: contributed important reagents.

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