Title Page

Accelerated telomere reduction and hepatocyte senescence in tolerated human liver allografts

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ABSTRACT

Background.

In living donor liver transplantation, the biological organ age of the donated allograft is unknown in young patients who receive grafts from older donors. Few studies have focused on the effects of aging on allografts in the state of tolerance. The purpose of this study was to assess the biological organ age of liver grafts.

Methods.

In 20 tolerated allografts over a 10-year post-transplant follow-up period, the relative telomere lengths were measured by multiplex quantitative polymerase chain reaction, and hepatocyte nuclear size and cell cycle phase markers were determined by immunohistochemistry. The results were compared with the same measurements that had been obtained prior to transplantation in the recipients' pre-implantation donor livers. Tolerance was defined strictly as a condition in which the allograft functioned normally and showed normal histology without any histological signs of rejection, fibrosis or inflammation in the absence of immunosuppression.

Results.

First, telomere length correlated with chronological donor age (n=41). Accelerated telomere reduction was seen in tolerated grafts compared with the predicted telomere length of each allograft calculated from the regression line of donor livers. Tolerated grafts were associated with higher hepatocyte p21 expression and greater nuclear area than in the donor livers prior to transplantation.

Conclusions.

These findings suggest that allografts age more rapidly than in the normal population, and that grafts may reach the limit of proliferative capacity even in the state of tolerance.

Keywords

Tolerance, liver transplantation, real-time PCR, cyclin-dependent kinase inhibitor p21, hepatocyte nuclear area

Footnote

ALT, alanine aminotransferase; AST, aspartate aminotransferase; FFPE, formalin-fixed, paraffin-embedded; PCR, polymerase chain reaction; T-Bil, total bilirubin; T/S ratio; the relative ratio of telomere repeat signals (T) to single-copy gene signals (S)

1. INTRODUCTION

Telomeres are located on the ends of chromosomes and help maintain genomic integrity and stability. Telomeres consist of tandem (TTAGGG)n nucleotide repeats, and shorten with age [1]. In the setting of chronic liver disease (i.e., liver cirrhosis, viral hepatitis), telomere length has been shown to be significantly shorter than in normal livers of the same age [2–4]. Pediatric patients often receive grafts from their parents through living donor liver transplantation; the biological organ age of the donated allograft is unknown in young patients who receive grafts from older donors. We previously reported that the hepatocyte telomere signal intensity was significantly lower than that of the predicted decline according to age in the tolerated liver allograft as well as that in chronic rejection, as revealed by quantitative fluorescence *in situ* hybridization [5]. In a larger number of cases, we performed quantitative real-time polymerase chain reaction (PCR), and confirmed accelerated telomere shortening relative to the chronological graft age in tolerated grafts. Recently, it has been demonstrated that measurement of relative average telomere lengths can be accomplished by real-time PCR using a carefully designed pair of oligonucleotide primers [6]. It is possible that a significant proportion of liver transplantation recipients are tolerant [7–9]. Tolerance is a condition in which an allograft functions normally and lacks histological evidence of rejection in the absence of immunosuppression [10]. Tolerated grafts are good material for evaluating the biological organ age of grafts unaffected by inflammation and immunosuppression. Marked heterogeneity of hepatocyte nuclear area is a feature of aging as well as of advanced liver disease [11–12]. Increased hepatocyte nuclear area, telomere shortening, and p21 expression—all

markers of aging or cellular senescence—have been described in hepatocytes in non-alcohol-related fatty liver disease [11–13].

2. OBJECTIVE

The aim of the present study was to use quantitative real-time PCR to assess whether accelerated telomere reduction was seen in tolerated grafts compared with telomere length as predicted by the chronological age of each allograft calculated from the regression line of donor livers. In addition, hepatocyte nuclear area and cell cycle phase markers were assessed morphologically and immunohistochemically. Graft aging was evaluated in 20 tolerated grafts in which the grafts functioned normally and lacked histological signs of rejection with no fibrosis or inflammation in the absence of immunosuppression.

3. MATERIALS AND METHODS

3.1. Definition of tolerance state

Transplantation tolerance has long been clinically defined as graft acceptance without functional impairment together with sustained acceptance for years in the absence of immunosuppression [7, 10]. This status has been called operational tolerance [14]. Analyses of protocol liver biopsies performed during long-term follow-up of liver transplant recipients who are tolerant have revealed a high frequency of graft fibrosis, albeit with the grafts showing normal liver function, compared with the grafts of patients on maintenance immunosuppression [15-17]. Thus, in the present study, tolerance was defined strictly as a condition in which the allograft functioned normally

and showed normal histology without any histological sign of rejection, fibrosis or inflammation in the absence of immunosuppression.

3.2. Study population

From 1990 to December 2012, 798 pediatric patients (≤18 years of age at liver transplantation) underwent living donor liver transplantation at Kyoto University Hospital using donor livers from their parents. There were 393 patients who were followed for more than 10 years after liver transplantation; 227 of those patients underwent a total of 598 biopsies at more than 10 years post-transplant. Of these, 70 patients showed normal histology, with 28 patients off immunosuppression. Thus, 12% (28/227) of pediatric patients at more than 10 years' follow-up were able to withdraw from immunosuppression, resulting in a state of tolerance.

For 6 of the 28 tolerant patients, DNA from paraffin-embedded sections of their donor livers was highly degraded, resulting in poor PCR amplification; thus, no further analysis could be performed. For another 2 patients, preserved samples of their donor livers at the time of transplantation were not available; therefore, those 2 patients were excluded from analysis. The remaining 20 patients were subjects for telomere length analysis.

This study was approved by the Kyoto University Institutional Review Board (G553).

3.3. Histological analysis

Histological analysis was performed on liver needle biopsy samples obtained at last follow-up. The donor livers (time zero biopsies) served as controls; these had been fixed in formalin and embedded in paraffin. All specimens were interpreted by pathologists

(AM-H and HH) on routine hematoxylin and eosin (HE) staining, Masson's Trichrome for evaluation of fibrosis, and immunohistochemistry for cytokeratin 7 (OV-TL12/30 DakoCytomation, Glostrup, Denmark; 1:300) in the bile duct epithelium.

3.4. Laboratory analysis

Postoperative clinical data were collected retrospectively. Laboratory data at the time of protocol biopsies for tolerant patients included the following variables: serum aspartate aminotransferase (AST, normal range, 13-29 IU/L), alanine aminotransferase (ALT, 8-28 IU/L) and total bilirubin (T-Bil, 0.2-1.0 mg/dL). The incidence of biopsy-proven acute rejection, other complications such as biliary and vascular complications during the follow-up period, and the amount of time off immunosuppression were also recorded.

3.5. Telomere length analysis by quantitative real-time PCR

Genomic DNA was extracted from the archived paraffin-embedded liver tissue using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Venlo, Netherlands). Telomere length was measured using a multiplexed quantitative real-time PCR method previously described by Cawthon on a real-time PCR cycler (Rotor-Gene Q, QIAGEN). In brief, telomere length value was determined by the relative ratio of telomere repeat signals (T) to single-copy gene signals (S) in experimental samples compared with a reference DNA sample (T/S ratio). The single copy gene used in the study was *36B4*, which encodes acidic ribosomal phosphoprotein PO located on chromosome 12. The telomere PCR experiments and the *36B4* PCR experiments were performed in triplicate [18]. The comparative threshold cycle method ($\Delta\Delta$Ct) was employed as the method of choice to

quantify relative gene expression. The quantification result was transformed to an exponential value $2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle.

3.6. Immunohistochemistry and hepatocyte nuclear area measurement

Monoclonal antibody for p21 was used as a marker of cell cycle arrest (SX118, DakoCytomation; 1:50). The BenchMark ULTRA Slide Staining System (Roche Diagnostics Japan, Tokyo, Japan) was used for the performance of automated immunohistochemistry. Hepatocyte nuclear p21 reactivity was assessed in a quantitative manner. The number of positive hepatocyte nuclei divided by the total number of hepatocytes counted on a biopsy specimen equaled the calculated index (%).

Hepatocyte nuclear area was recorded simultaneously on p21 staining slides for which the nuclear counter stain was hematoxylin. NIS-Elements D Microscope Imaging Software (Nikon Instruments Inc., Tokyo, Japan) was used to measure the nuclear area of hepatocytes. For each slide, at least 1000 hepatocytes were measured for nuclear size.

3.7. Statistical analysis

For analysis of clinical data, a *t*-test or U-test was performed. Regression analysis was used to test relationships between quantitative variables. A P-value < 0.05 was considered significant. For statistical analysis, JMP Start Statistics version 9 was used (Statistical Discovery Software SAS Institute, Cary, NC, USA).

4. RESULTS

4.1. Clinical profiles of tolerated grafts

The clinical characteristics of 20 tolerant patients included in the study are summarized in Table 1. The median age at the time of liver transplantation was 1 year (range, 0-15). The original diseases in the 20 study patients were biliary atresia in 16, liver cirrhosis from unknown cause in 1, Budd-Chiari syndrome in 1, fulminant hepatic failure of unknown etiology in 1, and congenital biliary dilatation in 1. Seven of the patients received left or lateral grafts from their father, and 13 received grafts from their mother. The graft was ABO-blood-type identical in 17 patients, compatible in 2, and incompatible in 1.

The study patients were followed at a median of 13 years after transplantation (range, 10-20). All received tacrolimus as baseline immunosuppression. Weaning was intentionally performed in 14 patients in a gradual manner. Two of the patients were noncompliant with respect to their immunosuppressant medication, and 4 had stopped immunosuppression due to infection. The median time off immunosuppression was 6 years (range, 2-18). Similarly, the duration of on immunosuppression was a median of 8 years (range, 2–17 years). Laboratory data for the patients who were tolerant at the time of the last biopsy revealed a median AST of 23 IU/L (range, 15-22), ALT 19 IU/L (range, 12-33), and T-Bil 0.7 mg/dL (0.4-1.4).

Among the 20 patients, 8 (40%) experienced at least 1 episode of acute cellular rejection at a median of 29 days (range, 9-539) post-transplant.

4.2. Telomere length in donor livers

As reference data, regression analysis of relative telomere length (T/S ratio, telomere repeat signals (T) to single-copy gene signals (S)) was performed using normal donor livers of the study population (n=41). The median age of the 41 donors was 35 years (range, 26-66 years), of which 16 were male and 25 were female. None of the donor livers histologically showed steatosis, fibrosis, necrosis, or inflammation.

We analyzed whether there was a statistically significant reduction rate in relative telomere length between the male donors (n = 16) and female donors (n = 25) using analysis of covariance (ANCOVA). We found that the slope parameters in the regression lines of the two groups were consistent with the parallel lines in our method (P= 0.081). Thus, we used regression lines that included all donor livers from both males and females.

Telomere length showed a negative correlation with age (R^2=0.136, P<0.001; Figure 1A).

4.3. Telomere shortening in tolerated allografts

The relative telomere lengths (T/S ratio) of the recipients' liver allografts were assessed at time zero (donor liver) and at the last biopsy at a median of 13 years (range, 10-20) post-transplant (Figure 1B). The predicted telomere length (T/S ratio) of each allograft at the last biopsy was calculated using the telomere length of each donor liver and the annual rate of telomere shortening (-1.13) of the reference line of donor livers. The results showed that the telomere length declined significantly relative to the predicted telomere length of the allograft (P<0.001; Figure 1C). We tried to analyze the relationship between the degree of telomere reduction and the duration of immunosuppressive treatment, or the duration off immunosuppression, but no correlation was found (n = 15; 5 patients had no information when immunosuppression

was stopped). A greater annual rate of telomere reduction (> 1.13) has not been associated with duration on immunosuppression (the association with > 5 years on immunosuppression; $P = 0.63$, > 10 years on immunosuppression; $P = 0.26$).

4.4. Hepatocyte nuclear area and p21 immunohistochemistry

Donor hepatocyte nuclear area increased with age (n=41) (R^2=0.03, P<0.001; Figure 2A). There were marked polymorphisms of the hepatocyte nuclear area in the tolerant patients (Figure 2B). The mean hepatocyte nuclear area was larger in the tolerated allografts at a median of 13 years post-transplant than in the donor livers (69.0 um^2 vs. 64.3 um^2, P=0.006). The predicted hepatocyte nuclear size of each tolerated allograft was calculated using the reference line of donor livers. The hepatocyte nuclear size of the tolerated allografts was significantly larger relative to the predicted nuclear size (P=0.003; Figure 2C).

No hepatocyte expression of p21 was seen in the donor livers, consistent with previous reports [13, 19]. In contrast, higher expression of hepatocyte p21 was seen in the tolerated allografts; the mean number of hepatocytes counted on each biopsy was 6783 ± 2992, and the mean number of p21-positive hepatocytes was 22.6 ± 22.2; thus, the mean proportion of p21-positive hepatocytes in the tolerated allografts was 0.42% ± 0.53% compared with no p21-positive hepatocytes in the donor livers (P=0.001). P21 expression was localized predominantly to hepatocyte nuclei of the periportal area (Figure 2D).

4.5. Association of telomere reduction, p21 staining, and hepatocyte nuclear area

There was a tendency for a greater annual rate of telomere reduction (>1.13) to be associated with episodes of acute cellular rejection; however, this finding was not statistically significant (P=0.068). We further tried to analyze the relationship between the number of episodes of acute rejection and the annual reduction rate of telomere shortening and found no significant correlation between them ($Y = 0.77 + 0.067X$, $R^2 = 0.01$, $P = 0.66$).

No significant correlation was found between telomere reduction and patient age, sex, or graft/recipient weight ratio. No significant correlation was found between p21 ratio and donor age, telomere reduction, or graft/recipient weight ratio. There was no difference in p21 ratio according to the presence or absence of acute cellular rejection.

5. DISCUSSION

We confirmed greater telomere shortening than predicted by chronological age of each allograft calculated from the regression line of donor livers in long-term transplanted liver allografts in the state of tolerance. This confirmed the results of our previous study using the method of quantitative fluorescence *in situ* hybridization [5]. Because reproducible quantitation to measure hepatocyte telomere length in formalin-fixed paraffin embedded liver tissue is technically difficult [20], we tried the other method (quantitative real time PCR) to confirm accelerated telomere shortening relative to the chronological graft age in a larger number of tolerated grafts. Loss of telomeric repeats (TTAGGG)n during sequential replications leads to cellular senescence [21]. In normal cells, activation of the DNA damage response machinery due to dysfunctional telomeres activates the G1 DNA damage checkpoint, upregulates p21/p16, and subsequently leads to senescence [22]. Senescence-inducing signals usually engage either the p53- or the p16-retinoblastoma protein (pRB) tumor suppressor pathways. Active p53 establishes senescence growth arrest by inducing the expression of p21, a cyclin-dependent kinase inhibitor [22]. Cells in senescence are insensitive to external stimuli, but remain metabolically active and contribute to impaired tissue integrity and persistent inflammation [23]. Senescent cells undergo characteristic morphological changes, including increased nuclear size [22]. In the present study, senescence markers, hepatocyte p21 expression, and hepatocyte nuclear area were increased in tolerated grafts. We attempted to determine p16 expression using immunohistochemistry, but no expression was observed in the hepatocytes in any of the samples.

Cellular senescence limits regenerative capacity and has been associated with chronic allograft failure in kidney transplantation [24-27]. The majority of studies regarding graft senescence have been for the kidney rather than the liver. The liver indeed has much stronger regenerative power than the kidney. However, many insults may reduce hepatic functional reserve, since sustained cellular turnover in chronic liver disease accelerates cellular senescence [2-4, 11-13]. In the field of liver transplantation, the undesirable prognosis of liver transplantation from aged donors has been well recognized [28]. Graft survival for small-for-size hepatic allografts (graft-to-recipient weight ratio <0.8%) from older-age donors was found to be significantly lower than for allografts from younger-age donors, suggesting that inability of older grafts to expand to meet the functional demands of recipients [29]. The principal problem of older donor tissue is its lower ability to withstand stress and repair. Pre-existing aging may reduce repair and survival capacity, and post-transplant stress (e.g., rejection) uses up even more of this capacity, leading to graft failure [30]. To the best of our knowledge, rejuvenation of the graft liver in humans has not been reported. A recent study has shown that an adult grafted liver did not appear to rejuvenate in a pediatric recipient, as assessed by immunohistochemical staining for senescence marker protein-30 [31]. The results of the present study suggested that even tolerated grafts might undergo a lowering of renewal capacity and a decrease in function as the recipients become older, although telomere length in normal livers has been observed to vary widely in individuals [2]. According to our study, the allograft could be a mean of 7.82 years older than the predicted age of the allograft (16.5–7.62/1.13 = 7.82 year) at a mean of 14.62 years post-transplant.

In a rat model, both allogeneic and syngeneic transplants have been characterized by shortened telomeres during ischemia at transplantation [24]. Ischemia and reperfusion during transplantation result in a transient increase of reactive oxygen species in the organ, which are potent inducers of DNA breaks. Oxidative DNA damage accelerates telomere shortening [21]. Our previous report suggested that accelerated telomere shortening occurs within the first year post-transplantation [5]. The telomere decline is probably due to premature aging of the graft that might occur during ischemia-reperfusion injury or graft regeneration immediately after transplantation [32]. Thus, telomere shortening in tolerated grafts could reflect not only the proliferative history of a cell, but also the accumulation of oxidative damage during the early post-transplant period [21]. Furthermore, oxidative DNA damage increases susceptibility to hepatic polyploidy, resulting in nuclear enlargement of hepatocytes [33]. Since cellular senescence may also be accelerated by the transplantation process, in both young and old tissues, modification of peri- or post-transplantation environmental stress may be possible to reverse aging factors. In our study, there was a tendency for a greater rate of telomere reduction with episodes of acute rejection: telomere attrition may occur at those times in addition to during the peri-transplantation period. In practical terms, the frequency of post-transplant events (e.g., rejection) should be reduced to prevent additional cell turnover. Moreover, graft pretreatment for protecting the liver from ischemia/reperfusion injury has been found to improve graft function in animal models [34] and it may be potential therapeutic target.

The limitation of the present study was that because whole liver homogenates were used, distinct intrahepatic cell lineages could not be assessed separately. Telomere length in whole liver homogenates might be unlikely to reflect hepatocyte telomere length, although most of the cells in liver tissue are hepatocytes [35]. In the current study, because of the difficulty in avoiding lymphocytes in the liver acini, only tolerated grafts without any inflammation during the long term after transplantation were selected. The liver biopsies during the early post-transplant period were performed mainly for acute cellular rejection or cholangitis; therefore, there were no histologically normal biopsies in the early period. Furthermore, the present study could not answer the question of whether telomere attrition might take place when patients were not tolerant to their grafts or whether telomere attrition continues at an increased rate in tolerant patients because only two points were examined (samples at transplantation and at last biopsies in the state of tolerance).

The six patients showing opposite correlations (2 for telomere length and 4 for hepatocyte nuclear size) were different in the two figures. One explanation for the discrepancy was the use of formalin-fixed, paraffin-embedded (FFPE) tissue for DNA analysis (telomere length). Obtaining DNA for molecular analysis for FFPE tissue is a challenge, since DNA from FFPE tissue is often scarce, degraded, and of low quality. FFPE tissues stored for long periods have shown a lower rate of amplification in PCR analysis than recent FFPE samples [36]. This may explain the opposite correlations in telomere length in the 2 cases. The discrepancy in hepatocyte nuclear size may have been due to counting hepatocytes in one needle biopsy specimen, which may not represent the whole liver allograft.

In conclusion, telomeres were shorter than expected for graft age in the long-term surviving tolerated liver allografts analyzed by quantitative real-time PCR. The tolerated grafts were also associated with higher hepatocyte p21 expression and greater nuclear area than in the donor livers. It is necessary of taking better care of an older liver to lessen possibility of rejection and further damage.

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Table 1. Clinical and demographic data for tolerant patients

	Tolerant patients (n=20)
Median age at transplantation (years)	1 (range, 0-15)
Median donor age (years)	32 (range, 28-50)
Sex (male/female)	(7/13)
Median time from transplantation (years)	13 (10-20)
Median time off immunosuppression (years)	6 (2-18)
Reason for immunosuppression withdrawal (n)	Intentionally performed (14)
	EBV infection (3)
	Noncompliance (2)
	Otitis media (1)
Liver function tests at last biopsy (median, range)	
AST (IU/L)	23 (15-22)
ALT (IU/L)	19 (12-33)
Total bilirubin (mg/dL)	0.7 (0.4-1.4)

EBV, Epstein-Barr virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase

Figure legends

Figure 1: Telomere length analyzed by real-time quantitative polymerase chain reaction (PCR).

(A) Telomere length of reference donor livers plotted against age in years (n=41). Regression analysis of the donor livers revealed age-dependent decline of relative telomere length (T/S ratio) measured by real-time PCR ($Y = 71.30 - 1.13X$, $R^2 = 0.136$, $P<0.001$). The regression line is shown as a solid line.

(B) Real-time PCR amplification curves for telomere length in donor livers (red curves) and tolerated liver allografts (blue curves) in the representative case (triplicated reactions). Donor DNA samples shown to have longer telomeres than tolerated allografts.

(C) Comparison of relative telomere length between the tolerated allografts at 10 years after liver transplantation and the predicted length of each allograft calculated from the regression line of the recipients' donor livers (n=20). The T/S ratio of the tolerated allografts was lower than the predicted T/S ratio of each allograft ($P<0.001$). Each line represents one patient.

Figure 2: Hepatocyte nuclear area measurement and p21 immunohistochemistry.

(A) In the reference donor livers, hepatocyte nuclear area was positively correlated with age (n=41) ($Y = 57.68 + 0.13X$, $R^2 = 0.03$, $P<0.001$).

(B) Measurement of hepatocyte nuclear area on a HE stained image in tolerated grafts by NIS-Elements D Microscope Imaging Software (Nikon Instruments Inc., Tokyo, Japan).

(C) The hepatocyte nuclear size of the tolerated allografts was larger than the predicted nuclear size calculated from the reference line of the recipients' donor livers (n=20). Each line represents one patient.

(D) Immunohistochemistry for p21 showing hepatocyte nuclear p21 expression (arrow) in the tolerated grafts (original magnification, x400). P indicates a portal area.

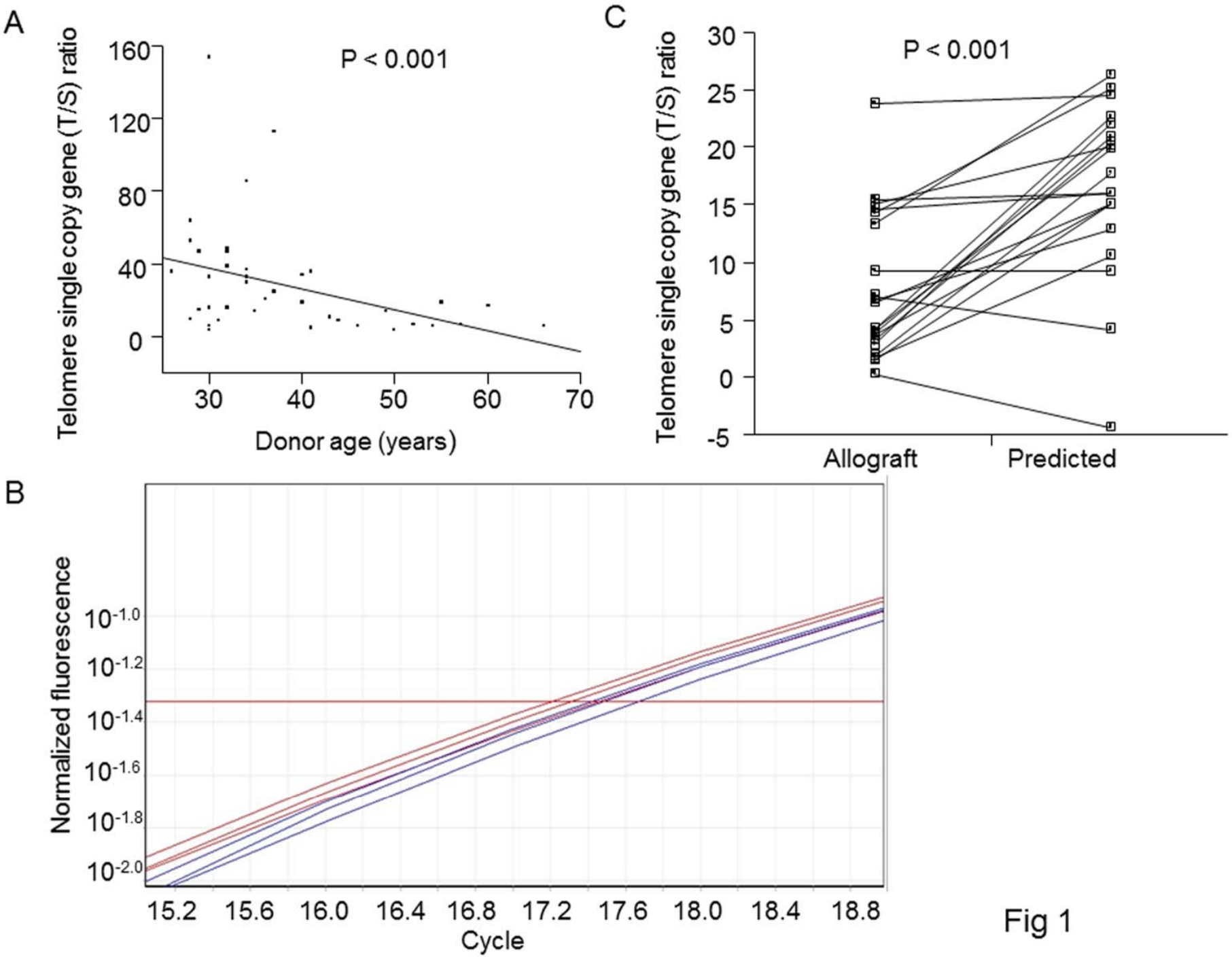
A
Telomere single copy gene (T/S) ratio
160
120
80
40
0
P < 0.001
30
40
50
60
70
Donor age (years)
C
Telomere single copy gene (T/S) ratio
30
25
20
15
10
5
0
-5
P < 0.001
Allograft
Predicted
B
Normalized fluorescence
10-1.0
10-1.2
10-1.4
10-1.6
10-1.8
10-2.0
15.2
15.6
16.0
16.4
16.8
17.2
17.6
18.0
18.4
18.8
Cycle

Fig 1

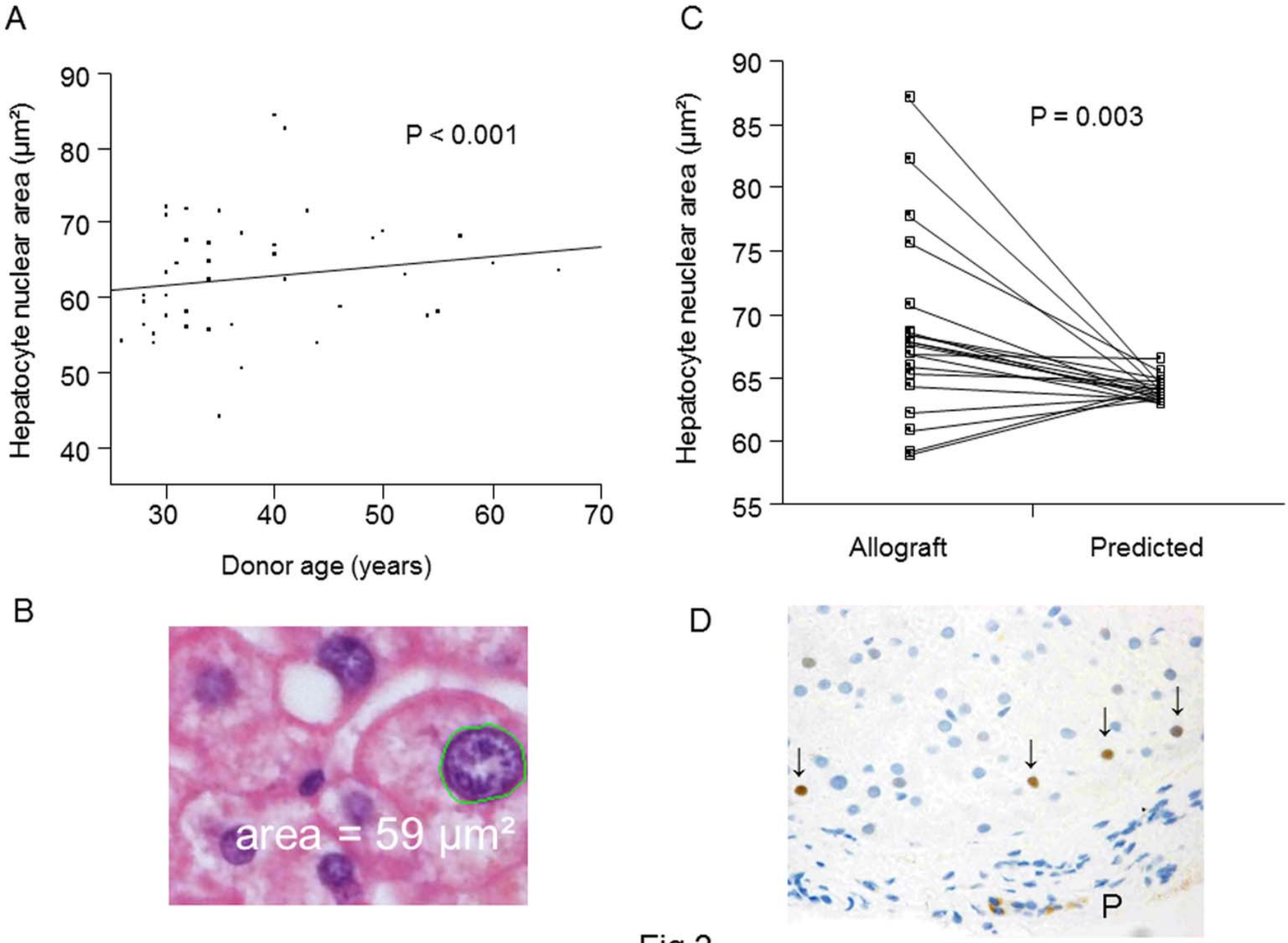
A
Hepatocyte nuclear area (µm²)
90
80
70
60
50
40
P < 0.001
30
40
50
60
70
Donor age (years)
C
Hepatocyte neuclear area (µm²)
90
85
80
75
70
65
60
55
P = 0.003
Allograft
Predicted
B
area = 59 µm²
D
P

Fig 2