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ORIGINAL ARTICLE

Up-regulated LRRN2 expression as a marker for graft quality in living donor liver transplantation

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Abstract

The quality and size of liver grafts are critical factors that influence living-donor liver transplantation (LDLT) function and safety. However, the biomarkers used for predicting graft quality are lacking. In this study, we sought to identify unique graft quality markers, aside from donor age, by using the livers of non-human primates. Hepatic gene microarray expression data from young and elderly cynomolgus macaques revealed a total of 271 genes with significantly increased expression in the elderly. These candidate genes were then narrowed down to six through bioinformatics analyses. The expression patterns of these candidate genes in human donor liver tissues were subsequently examined. Importantly, we found that grafts exhibiting up-regulated expression of these six candidate genes were associated with an increased incidence of liver graft failure. Multivariable analysis further revealed that up-regulated expression of *LRRN2* (encoding leucine-rich repeat protein, neuronal 2) in donor liver tissue served as an independent risk factor for graft failure (odds ratio 4.50, confidence interval 2.08–9.72). Stratification based on graft expression of *LRRN2* and donor age was also significantly associated with 6-month graft survival rates. *Conclusion*: Up-regulated *LRRN2* expression of liver graft is significantly correlated with graft failure in LDLT. In addition, combination of graft *LRRN2* expression and donor age may represent a promising marker for predicting LDLT graft quality.

Takahiro Tomiyama and Shokichi Takahama contributed equally to this work.

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INTRODUCTION

Liver transplantation (LT) is the primary curative therapy for patients with decompensated liver cirrhosis, which has a poor prognosis. Although LT provides long-term survival benefit[1] to these patients, organ shortage is a primary obstacle as the demand for grafts exceeds the number of available allografts.[2] To overcome this issue, living donor LT (LDLT) was established in 1989.[3,4] Currently, the three major factors influencing successful LDLT are graft size, graft quality, and recipient condition, particularly within patients with chronic liver disease or portal hypertension, which can lead to graft failure.[5] As such, there is a need to identify biomarkers capable of accurately predicting graft failure before transplantation.[6,7]

We previously reported useful biomarkers for predicting LDLT graft failure, including a model for end-stage liver disease (MELD) score, the presence of a portosystemic shunt, graft volume-to-standard liver volume (GV/SLV) ratio, and donor age.[8] The biomarkers that correlate with graft quality for LT include steatosis, cold ischemic time (CIT), and donor age.[9–11] Moreover, short-term combination therapy involving diet, exercise, and medications can improve the health of LDLT donors. Currently, unlike LT, donor age is the most commonly used biomarker for LDLT graft quality. Interestingly, the number of Kupffer cells in the liver—defined by CD68 expression—decreased in elderly donors (≥50) and was correlated with patient survival, suggesting that grafts from elderly individuals can be further stratified by quality, characterized by the cellular components or gene/protein expression profiles.[12] Thus, gene-expression profiling of the graft has the potential to identify alternative markers for graft quality in the elderly.

The process of aging is influenced by multiple factors, including smoking, eating habits, and metabolic status. While age is an indicator of graft quality, it is a multifactorial metric in the presence of many confounding variables; therefore, age is correlated with successful grafting in some patients but not in others.[11] Hence, investigating the factors in the liver that change with age and how such factors affect LT is warranted. Donor age is expected to increase with increased life expectancy of the general population; hence, the selection of good-quality grafts from older donors will become more urgent.

Several functional and genetic changes reportedly occur in the liver with aging. In particular, mitochondrial function, which is essential for liver regeneration after resection, declines with age in mammals.[13,14] Moreover, a previous study using single-cell RNA sequencing demonstrated that the number of differentially expressed genes (DEGs) significantly increases with age in most organs, including the liver.[15] Although genetic alterations associated with aging have been comprehensively analyzed in small animal models, aging-related genetic changes in human organs used for transplants, such as the liver, heart, and kidneys, remain poorly understood.

Non-human primates are considered one of the best preclinical models due to their genetic, physiological, and anatomical similarity to humans compared with rodents. Cynomolgus macaques (*Macaca fascicularis*) have been used as the clinically relevant experimental model for transplantation.[16] For instance, a nonmyeloablative preparative regimen for kidney transplantation in cynomolgus macaques was successfully translated to a clinical study in kidney patients.[17] Furthermore, the livers of these non-human primates are more anatomically similar to humans compared with small animals (e.g., rodents) or large non-primate mammals, such as pigs or dogs.[18] In addition, non-human primates, including the cynomolgus macaque (*M. fascicularis*), have been established models for human aging or age-related diseases in various research fields.[19,20]

Thus, we hypothesized that using cynomolgus macaques would minimize the effects of confounding factors on aging and facilitate the analysis of the effects of age-related genetic changes on graft quality.[21,22] Our primary aim was to identify graft quality markers associated with aging in liver tissue using a cynomolgus macaque experimental model to investigate their roles in determining graft quality for LDLT.

EXPERIMENTAL PROCEDURES

Study design and patients

We retrospectively reviewed data for 350 patients with chronic liver failure who underwent LDLT at Kyushu University between May 2004 and May 2019 and whose liver tissue was preserved. Inclusion criteria included recipient with chronic liver disease or hepatocellular carcinoma. Exclusion criteria were age < 18 years, recipient death caused by suicide or traffic accident, and recipient

with acute liver failure. Preoperative blood tests were performed to prescreen for indicators of graft quality in the human liver donors. No significant associations with transplant success were observed (Table S1). The study protocol was approved by the Institutional Review Board of the Kyushu University Hospital, approval number 2019–354. This study was conducted according to the Declaration of Helsinki of 1996. Written informed consent was obtained from all patients before LDLT.

Animal experiments

In total, 13 (five males and eight females) cynomolgus macaques (*M. fascicularis*), which tested negative for simian immunodeficiency virus, simian type D retrovirus, simian T cell lymphotropic virus, simian foamy virus, Epstein–Barr virus, cytomegalovirus, and B virus, were used in this study. They are estimated to age approximately 3 times more rapidly than humans.[23] Therefore, considering that the main cutoff age for human donors is 50 years,[5] we set 17 years (equivalent to 51-year-old humans) as the cutoff for categorizing the old and young animals. Liver tissues were collected from the lower right lobe, snap-frozen in 1.5-ml tubes by liquid nitrogen, and stored at −80°C. All animal studies were performed at Tsukuba Primate Research Center, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN). The animal studies were approved by the Committee on the Ethics of Animal Experiments of NIBIOHN and performed according to the guidelines for animal experiments at NIBIOHN. The animals were used under veterinary supervision.

Assessment of early graft failure

Early graft failure was defined as the occurrence of small-for-size-graft (SFSG) syndrome or graft loss within 6 months of LDLT, excluding cases with acute or chronic rejection and hepatitis C virus recurrence. SFSG syndrome was defined based on the following criteria: serum total bilirubin concentration ≥10 mg/dl on day 14 following LDLT; amount of ascites and pleural effusion >1000 ml on day 14 following LDLT and 500 ml on day 30 following LDLT[24]—a requirement for frequent or sustainable puncture for pleural and abdominal effusion after 30 days following LDLT.

Quantitative real-time polymerase chain reaction

RNA extraction, from human or cynomolgus macaque liver tissue, was performed using the Maxwell RSC RNA cells and RNA tissue kit (Promega Co.). Complementary DNA (cDNA) was synthesized using a SuperScript cDNA synthesis kit (Invitrogen). PCR was performed using the StepOnePlus Real-Time PCR system (Applied Biosystem Inc.) with a QuantiTect SYBR Green PCR kit (Qiagen). The polymerase chain reaction (PCR) conditions were 35 cycles of 15 s at 95°C and 60 s at 60°C. Primer sequences are listed in Table S2. The relative expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. All measurements were obtained twice as technical replicates.

Immunohistochemistry

Detection of LRRN2 protein was performed using 4-μm-thick formalin-fixed and paraffin-embedded sections. Sections were deparaffinized, pretreated with Target Retrieval Solution (Dako) in a decloaking chamber at 110°C for 15 min, and incubated with 10% hydrogen peroxidase in methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. Next, the sections were incubated with anti-LRRN2 rabbit polyclonal antibody (catalog no. HPA029124; Sigma Aldrich; 1:200) at 4°C overnight. The immune complex was detected using the Dako EnVision detection system. Sections were finally incubated with 3,3′-diaminobenzidine (DAB), counterstained with hematoxylin, and mounted. Raw images (ndpi files) were processed by Fiji (version 2.3.0) with NDPITools. Imported stack images were processed using the functions “stack to RGB,” then “color deconvolution” with an option of “H + DAB,” to calculate the intensity of DAB staining. Details are described in Figure S4.

Statistical analysis

Categorical variables, presented as numbers and percentages, were compared using Pearson's chi-square test. Based on their distributions, continuous variables were presented as medians with ranges and compared using Student's *t* test. The median observation period was 5.56 (range 0.02–16.1) years. Continuous and categorical variables were compared using Student's *t* test and χ^2 test, respectively. Graft survival data were analyzed using the Kaplan–Meier method and compared using the log-rank test. Bonferroni adjusted *p* value ($p = 0.0083$) threshold was considered to indicate a significant predictor in the analysis of graft survival in Figure 3, and early graft failure in Table 1. Receiver operating characteristic (ROC) curves and Youden's index were used to determine the cutoff, sensitivity, and specificity values for the gene expression and characteristics of patients with early graft failure. Univariable and multivariable analysis were performed with early graft failure as the dependent variable. Multivariable logistic regression was performed with backward elimination with all variables that were used in univariable

TABLE 1 Frequency of early graft failure stratified by gene expression

	Early graft failures/total in group (% graft failure)		
Gene	Low expression group	High expression group	*p* value
LRRN2	20 of 262 (7.63%)	21 of 88 (23.9%)	<0.0001
ZYX	25 of 286 (8.74%)	16 of 64 (25.0%)	<0.0001
ATP1A1	29 of 290 (10.0%)	12 of 60 (20.0%)	0.0283
LTBP4	28 of 313 (8.95%)	13 of 37 (35.1%)	<0.0001
PCED1B	16 of 207 (7.73%)	25 of 143 (17.5%)	0.0053
FOSB	28 of 314 (8.92%)	13 of 36 (36.1%)	<0.0001

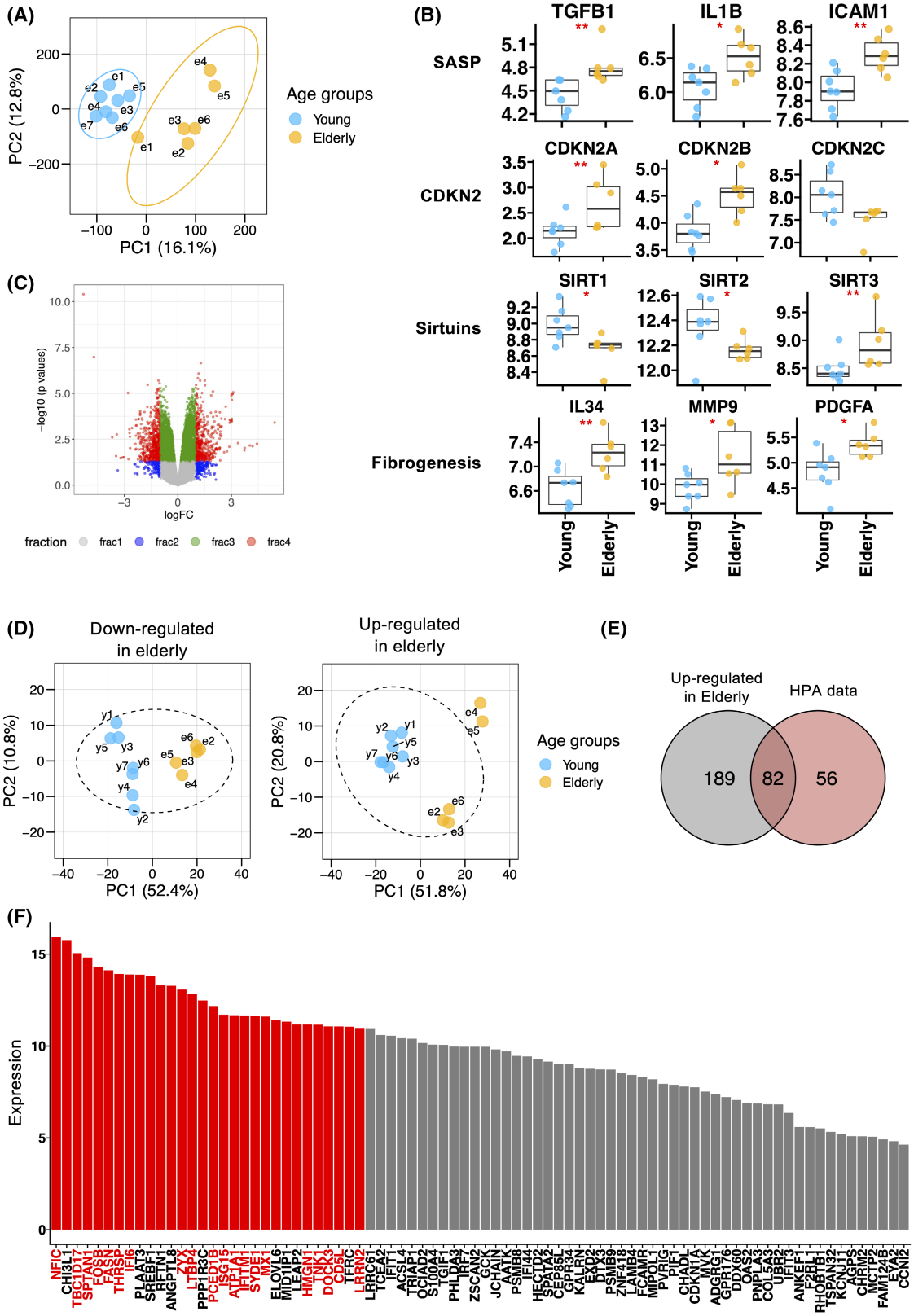
analysis. A multivariable analysis was performed along with clinical factors to assess the prognostic value of these six genes. Of the 350 patients, only 41 exhibited graft failure. Hence, the number of variables had to be limited for accurate multivariable analysis. ROC curves were generated for graft failure and individual gene expression. Genes with area under the curve (AUC) values < 0.6 were not included in multivariable analyses. Results are shown as odds ratios with 95% confidence intervals. A value of $p < 0.05$ was considered to indicate a significant predictor of early graft failure. All statistical analyses were performed using JMP Pro 15 software (SAS Institute) and R software version 3.6.2.

RESULTS

Identification of candidate genes for markers of qualitative differences in elderly monkeys

Based on the hypothesis that the gene-expression profile of the liver may reflect age-related liver quality, we performed a microarray analysis of cynomolgus macaque liver tissues to identify age-associated changes in gene expression. Cynomolgus macaques were divided into young (n = 7, 5–9 years old) and elderly (n = 6, 26, or 27 years old) groups (Tables S3 and S4). The expression of approximately 21,032 genes was successfully quantified for each macaque. Among the 58,839 probes covering 21,032 unique genes, 53,386 probes covering 17,200 genes were annotated by official symbols. To examine the age-related changes, we conducted principal component analysis (PCA) using all 51,256 probes and observed clear segregation of the young and elderly groups, suggesting the age-related changes in gene expression were obviously captured by this experiment (Figure 1A). To validate the data set for predicting liver aging, the expression of known aging/fibrosis marker genes was examined. Genes involving senescence-associated secretory phenotype (SASP) and cyclin-dependent kinase inhibitor 1A (CDKN2A; p16) were up-regulated in the elderly, while sirtuins (*SIRT*) were down-regulated, as also observed in human studies for SASP,[25] p16,[26] and *SIRT*.[27] Moreover, it is well known that liver fibrogenesis triggered by chronic damage is increased in the elderly.[28] Genes involved in liver fibrogenesis, such as interleukin (IL)-34, are also up-regulated in the elderly macaques,

FIGURE 1 Screening of graft quality markers for living donor liver transplantation using cynomolgus macaque liver tissue. (A) Principal Component Analysis (PCA) of cynomolgus macaque hepatic gene-expression microarray data. The circles indicate the probability ellipse. Colors indicate the age group. (B) Boxplots show the comparison of the aging-related gene expressions between young versus elderly groups. Each dot indicates the values obtained from individual macaques. Colors indicate the age group. Statistical significance is indicated by stars based on p values from Wilcoxon test (*$p < 0.05$, **$p < 0.01$). (C) Volcano plot shows the differentially expressed genes (DEGs) between young versus elderly groups. Colors indicate the fraction of the genes. (D) Scatter plots indicate the PCA of down-regulated genes or up-regulated genes. The circles indicate the 90% probability ellipse. Colors indicate the age group. (E) Venn diagram indicates the overlap of the two gene lists: one from up-regulated genes in the elderly and the other from hepatic expressed gene set obtained from human protein atlas. (F) The mean expression of 82 commonly observed genes in elderly macaques. Red bars indicate top 30 highly expressed genes. Red text indicates the top 20 high variance genes. *ATP1A1*, ATPaseNA+/K+ transporting subunit alpha 1; *CD5L*, CD5 molecule like; *CDKN*, cyclin-dependent kinase inhibitor; *CHI3L1*, chitinase 3 like 1; DOCK3, dedicator of cytokinesis 3; *ELOVL6*, ELOVL fattly acid elongse 6; *FASN*, fatty acid synthase; *FOSB*, FosB proto-oncogene, AP-1 transctiption factor subunit; *HMGN1*, high morbility group nucleosome binding domain 1; HPA, Human Protein Atlas; *ICAM1*, intercellular cell adhesion molecule 1; *IFI6*; interferon alpha inducible protein 6; *IFITM1*, Interferone Induced Transmembrane Protein 1; IL, interleukin; *ISG15*, ISG15 ubiquitin like modifer; *LEAP2*, liver enriched antimicrobial peptide 2, *LRRN2*, leucine rich repeat neuronal 2; *LTBP4*, latent tranforming growth factor beta binding protein 4; *MID1IP1*, MID1 interacting protein 1; *MMP*, matrix metalloproteinase; *MX1*, MX dynamin like GTPase 1; *NFIC*, nuclear factor I C; PC, principal component; *PCED1B*, PC-esterase domain containing 1B; *PDGFA*, platelet derived growth factor subunit A; *PLAAT3*, phospholipase A and acyltransferase 2; *PPP1R3C*, protein phosphatase 1 regulatory subunit 3C, *RFTN1*, raftlin, lipid raft linker 1; *ANGPTL8*, angiopoietin like 8; SASP, senescence-associated secretory phenotype; SIRT, sirtuin; *SPTAN1*, spectrin alpha non-erythrocytic 1, *SREBF1*, sterol regulatory element bingding transcription factor 1; *SYDE1*, synapse defective rho GTPase homolog1; *TBC1D17*, TBC1 domain family member 17; *TFRC*, transferrin receptor; *THRSP*, thyroid hormone responsive; *TGFB1*, transforming growth factor beta 1; *TNK1*, tyrosine kinase non receptor 1; *ZYX*, zyxin



as reported in advanced liver cirrhosis.[29,30] These data suggest that our model reflects changes in gene expression within aging cynomolgus macaques and humans (Figure 1B). Notably, to identify distinct differences between the young and elderly groups, one elderly macaque (e1) located at the margin of probability ellipse in the PCA plot was excluded from further analyses.

DEG analysis revealed that 468 genes (271 up-regulated and 197 down-regulated in the elderly group)

were differentially expressed (Figure 1C). The distribution of individual data points in the PCA plot of up-regulated genes was more scattered in the elderly group compared with the down-regulated genes, wherein each individual data point was located within the probability ellipse (Figure 1D). Thus, the up-regulated genes likely reflect qualitative individual differences, particularly among the elderly group.

To narrow down the gene sets based on the up-regulated genes, we used expression profiles for liver samples from the Human Protein Atlas (HPA). We selected 138 genes out of the total 468 DEGs based on the following two criteria: (1) confirmed protein expression by immunohistochemistry (IHC) in human liver; and (2) no protein expression data but exhibits high mRNA expression (Table S5A,B). We identified 82 overlapping genes between the 138 genes from HPA and the 271 up-regulated genes in this study (Figure 1E, Figure S1A). Furthermore, we hypothesize that genes with high variance may reflect the qualitative differences of aging, particularly in the elderly group. Hence, we selected the 20 most highly expressed genes (Figure 1H, Figure S1B) and found six genes (ATPase Na+/K+ transporting subunit alpha 1 [*ATP1A1*], FosB proto-oncogene (*FOSB*), *LRRN2*, latent transforming growth factor beta binding protein 4 [*LTBP4*], PC-esterase domain containing 1B [*PCED1B*], and zyxin [*ZYX*]) whose function is relatively unknown in the liver for the human study.

Correlation between candidate gene expression and donor age

The expression of the selected six candidate genes was examined in human clinical samples. The cohort included 350 live human livers. The clinical characteristics of the donors were median age = 36 years (range 20–63 years) and median MELD score = 15 (range 4–43). Meanwhile, the median GV/SLV and graft recipient weight ratios (GRWRs) were 40.6% (range 23.2%–73.1%) and 0.771% (range 0.430%–1.78%), respectively. In total, 289 recipients (82.6%) underwent simultaneous splenectomy.[6]

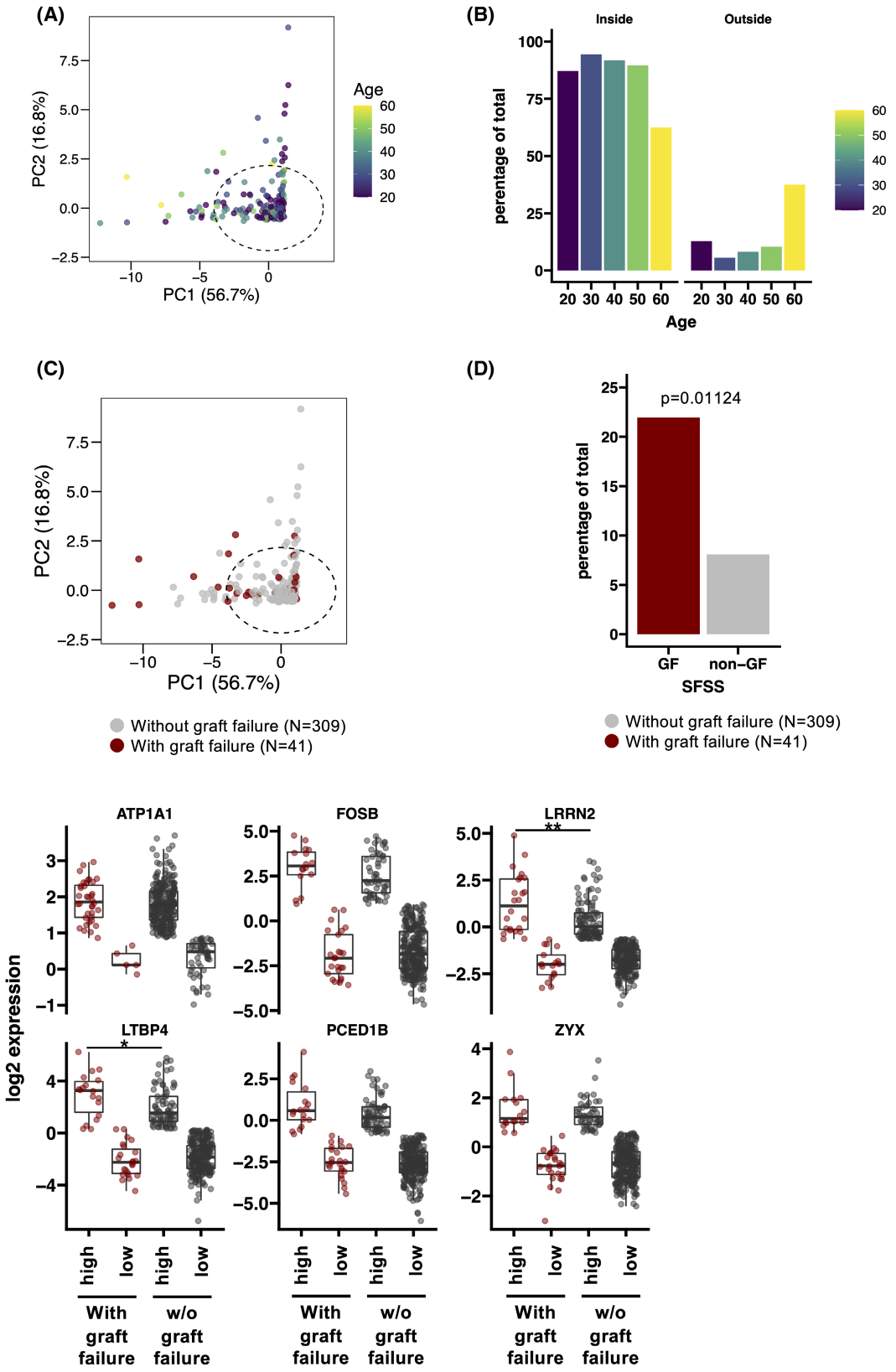
RNA was extracted from 350 livers, and the expression levels of the six candidate genes were analyzed by quantitative real-time PCR. Human samples were divided into elderly (≥50 years) and young (<50 years) groups (Table S6). The expression levels of all six genes were not significantly different between the groups. To examine whether the six candidate genes can differentiate donors based on age as observed in macaques, PCA was performed to determine the expression of the six candidate genes. Scatter plot analysis showed no distinct clustering; however, most donors were concentrated at the center of the 90% probability ellipse. The ratio of donors inside or outside the ellipse, based on the donor's age, revealed more elderly patient data points outside the 90% confidence ellipse compared with those of young donors (Figure 2A,B), suggesting that liver quality determined by the six candidate genes delineated based on aging.

Correlation between gene expression and early graft failure

To investigate the relationship between the expression levels of the six candidate genes and early graft failure, the patients were divided into two groups using the 90% probability ellipse, as the incidence of graft failure has been previously reported as about 10%.[5] Results show that recipients with grafts from donors outside the probability ellipse more frequently developed graft failure compared with those receiving grafts from donors inside the probability ellipse ($p = 0.01124$; Figure 2C,D).

Furthermore, to examine the contribution of each candidate gene for predicting graft failure, we plotted ROC curves for graft failure for each gene, set cutoff values, and divided donors into high and low expression groups (Figure S2A). The frequency of graft failure in the donors exhibiting up-regulated expression of five genes, excluding *ATP1A1*, was significantly higher than in donors exhibiting down-regulated expression (Table 1). Among the high-expression group, the expression levels of *LRRN2* and *LTBP4* were higher in donors with graft failure (Figure 2E), suggesting that the expression of these two genes could predict graft failure more accurately than others. These data demonstrated that graft failure is potentially predicted by the gene-expression status of the donors.

FIGURE 2 Expression levels of six candidate genes according to the early graft failure after living donor liver transplantation. (A) PCA of human donor hepatic expression levels for six candidate genes determined using quantitative real-time polymerase chain reaction. Black circles denote the 90% probability ellipse. Color of points indicates patient age group. (B) Percentage of donors inside or outside of the probability ellipse according to age. (C) PCA of human donor hepatic expression levels for six candidate genes determined using quantitative real-time polymerase chain reaction. Black circles denote the 90% probability ellipse. Dark red and gray points indicate patients with and without early graft failure, respectively. (D) Percentage of early graft failure in patients inside and outside the probability ellipse. Dark red and gray bars indicate the percentage of donors outside of the probability ellipse according to age. (E) Expression of six candidate genes in each group. The high expression group and low expression group were determined by the cutoff value based on the receiver operating characteristic (ROC) curve. Dark red and gray points indicate patients with and without early graft failure, respectively. Statistical significances between high groups are indicated by stars based on *p* values from Wilcoxon test (*$p < 0.05$, **$p < 0.01$). Abbreviations: *ATP1A1*, ATPase NA+/K+ transporting subunit alpha 1; *FOSB*, FosB proto-oncogene; GF, graft failure; *LRRN2*, leucine-rich repeat neuronal 2; *LTBP4*, latent transforming growth factor beta binding protein 4; *PCED1B*, PC-esterase domain containing 1 B; *ZYX*, zyxin



Additionally, the survival rates for recipients of grafts from donors exhibiting up-regulated expression of four genes, excluding *ATP1A1* and *PCED1B*, were lower than those whose donors exhibited down-regulated expression (Figure 3A–F). Patient characteristics as a function of the expression of each gene are listed in Table S7A–F. To narrow down the genes for multivariable analysis, the AUC was determined from the ROC curves (Figure S2A). Those genes with AUC values ≥0.6 were *LRRN2*, *ZYX*, and *PECD4*, which were used for univariable and

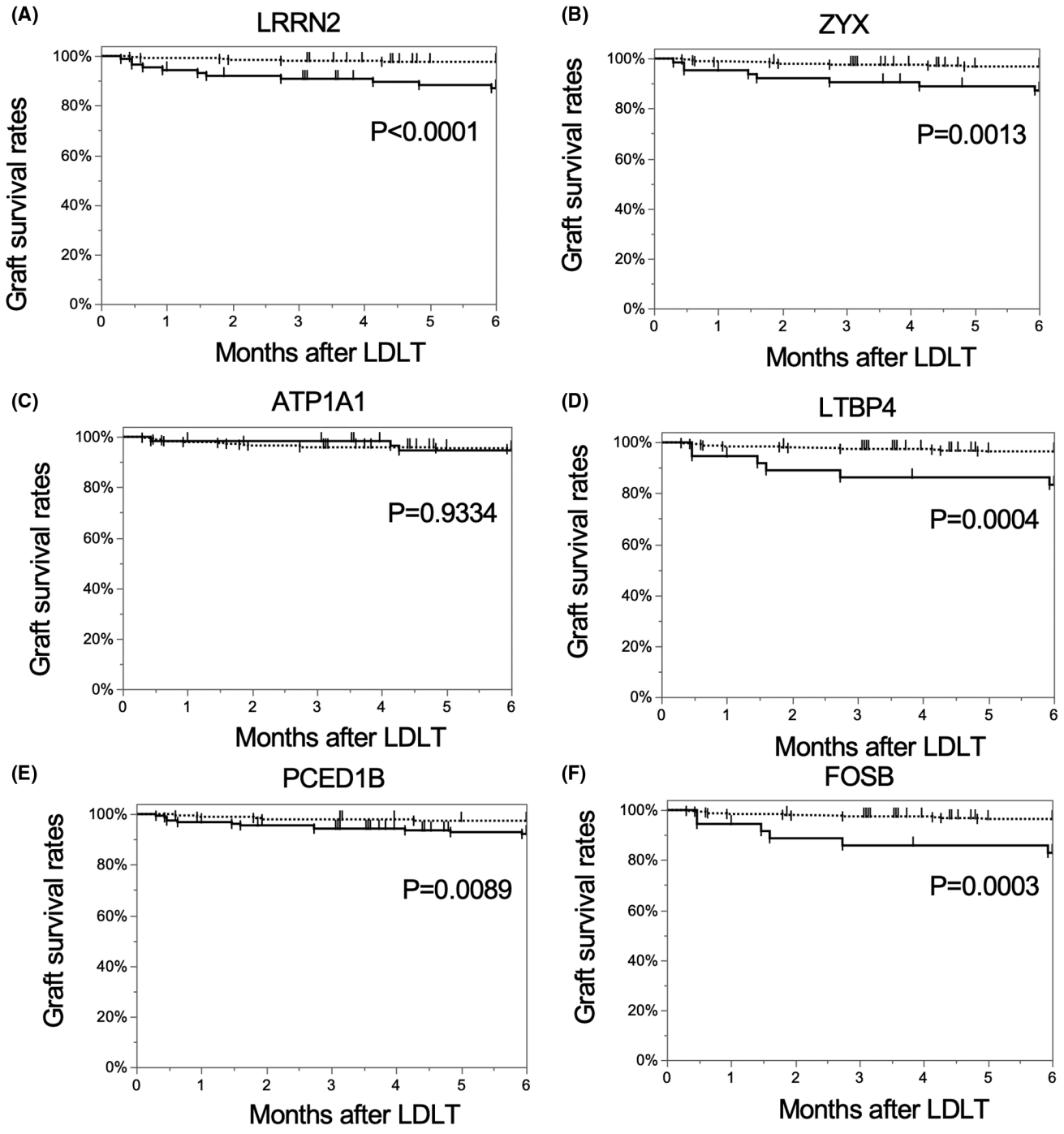


FIGURE 3 Analysis of early graft loss after living donor liver transplantation according to the expression levels of six candidate genes. *LRRN2* (A), *ZYX* (B), *ATP1A1* (C), *LTBP4* (D), *PCED1B* (E), and *FOSB* (F). LDLT, living-donor liver transplantation

multivariable analyses. Multivariable analysis indicated that donor age≥50 years, MELD score>20, actual GV/SLV<35% or GRWR <0.7%, absence of splenectomy, and high *LRRN2* expression were independent prognostic factors for graft failure (Table 2). To identify the confounding factors for multivariable analysis, we also calculated correlation coefficients and found no strong correlation between the genes, examining different age groups and all ages, respectively (Figure S3), suggesting that no confounding factors are impacting the three genes.

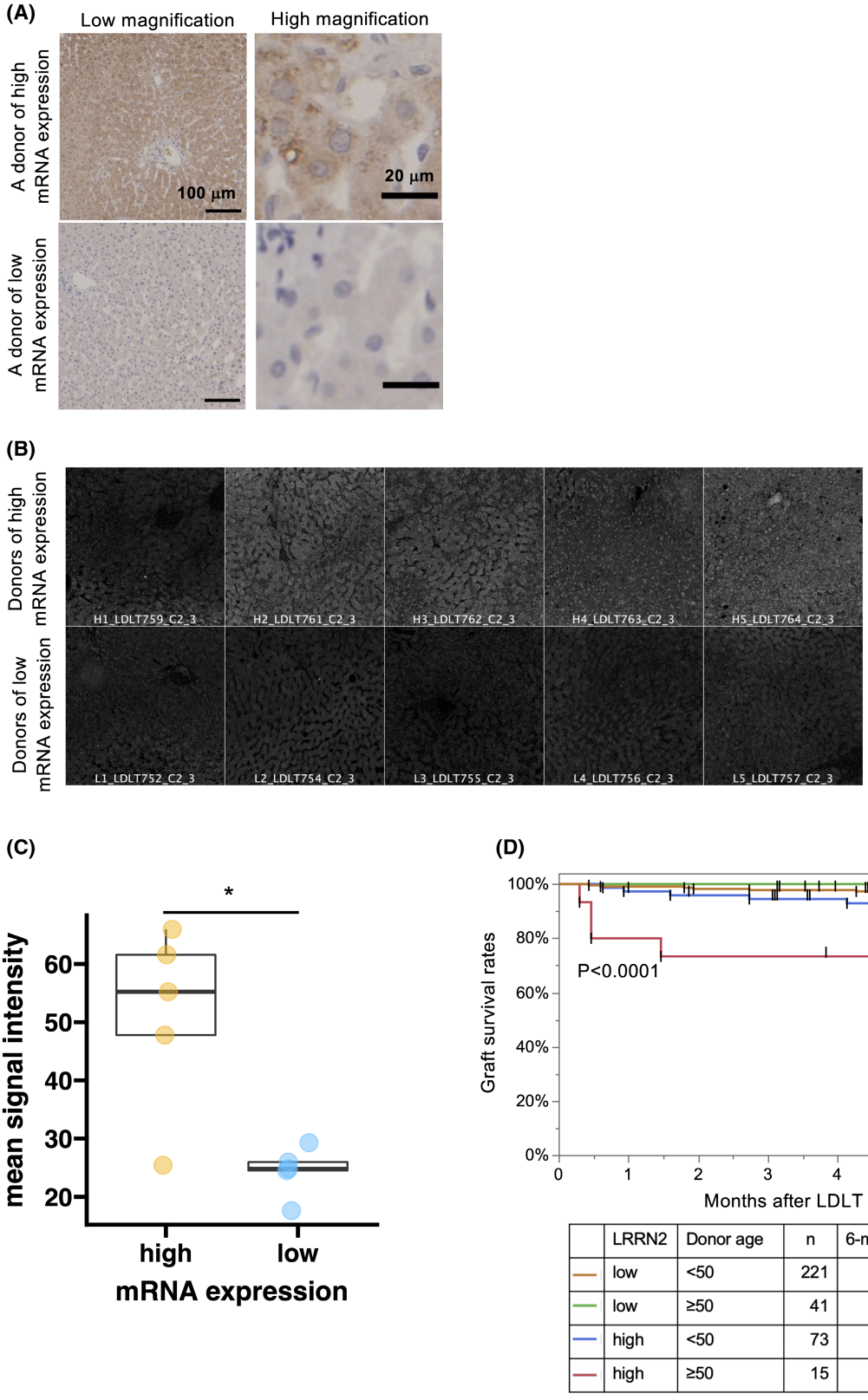
Combining LRRN2 expression with donor age to predict graft failure

LRRN2 was identified as the independent prognostic factor for graft failure; therefore, to further investigate its contribution in predicting liver graft failure, the expression of LRRN2 protein was examined in the two donor groups. IHC analysis revealed a clear LRRN2 signal in hepatocytes, with a stronger signal intensity within the hepatic tissues of donors in the high *LRRN2*

TABLE 2 Predictors of early graft failure

	Univariable analysis			Multivariable analysis		
Variables	**OR**	**95% CI**	***p* value**	**OR**	**95% CI**	***p* value**
Donor variables						
LRRN2						
Low (*n* = 262)	1	(reference)		1	(reference)	
High (*n* = 88)	3.79	1.94–7.41	<0.0001	4.06	1.89–8.70	0.0003
ZYX expression						
Low (*n* = 286)	1	(reference)				
High (*n* = 64)	3.48	1.73–7.00	0.0008			
PCED1B expression						
Low (*n* = 286)	1	(reference)				
High (*n* = 64)	2.32	1.18–4.56	0.0124			
Gender						
Male (*n* = 212)	1.14	0.584–2.25				
Female (*n* = 138)	1	(reference)	0.6919			
Age (year)						
<50 (*n* = 294)	1	(reference)		1	(reference)	
≥50 (*n* = 56)	2.15	1.01–4.60	0.0481	3.62	1.49–8.83	0.0046
Graft						
Right (*n* = 180)	1	(reference)				
Others (*n* = 170)	1.58	0.814–3.05	0.1737			
Actual GV/SLV (%) or GRWR (%)						
≥35 and≥0.7 (*n* = 227)	1	(reference)		1	(reference)	
<35 or <0.7 (*n* = 123)	1.9	0.988–3.67	0.0561	2.73	1.26–5.91	0.0108
ABO incompatible						
No (*n* = 297)	1	(reference)				
Yes (*n* = 53)	0.409	0.121–1.38	0.1488			
Recipient variables						
Gender						
Male (*n* = 171)	0.713	0.368–1.38				
Female (*n* = 179)	1	(reference)	0.3151			
Age (years)						
<50 (*n* = 90)	1	(reference)				
≥50 (*n* = 260)	0.937	0.448–1.96	0.8625			
Preoperative DM						
No (*n* = 291)	1	(reference)				
Yes (*n* = 59)	1.23	0.535–2.81	0.6347			
Hepatocellular disease						
No (*n* = 83)	1	(reference)				
Yes (*n* = 267)	0.959	0.449–2.05	0.9138			
MELD score						
≤20 (*n* = 280)	1	(reference)		1	(reference)	
>20 (*n* = 70)	3.02	1.51–6.04	0.0018	4.7	2.05–10.8	0.0002
Splenectomy						
With splenectomy (*n* = 289)	1	(reference)		1	(reference)	
Without splenectomy (*n* = 61)	3.75	1.86–7.58	0.0002	9.43	3.88–22.9	<0.0001

Abbreviations: GV/SLV, graft volume/recipient standard liver volume ratio; GRWR, graft recipient weight ratio; ICU, intensive care unit; DM, diabetes mellitus; MELD, Model for End-Stage Liver Disease.



	LRRN2	Donor age	n	6-month survival
	low	<50	221	97.3%
	low	≥50	41	100%
	high	<50	73	95.1%
	high	≥50	15	66.6%

FIGURE 4 Value of LRRN2 expression as a predictor of graft failure. (A) Representative immunohistochemistry (IHC) staining of LRRN2 in the live liver donor. IHC staining of LRRN2 in the donor exhibiting high *LRRN2* messenger RNA (mRNA) expression and low mRNA expression. (B) 3,3′-diaminobenzidine (DAB) signal data extracted from IHC images in the donor exhibiting high *LRRN2* mRNA expression and low mRNA expression (n = 5 each). (C) Relative staining intensity obtained from DAB signals in IHC staining of LRRN2 (n = 5 each). (D) Kaplan–Meier curves for early graft failure in recipients according to LRRN2 expression and donor age

mRNA expression group compared with the low expression group. These results agreed with the quantitative real-time PCR data (Figure 4A–C).

To investigate whether LRRN2-dependent prediction is age-related, recipients were divided into four groups: LRRN2 low/donor age < 50 years (n = 221); LRRN2 low/donor age ≥ 50 years (n = 41); LRRN2 high/donor age < 50 years (n = 73); and LRRN2 high/donor age ≥ 50 years (n = 15). We observed a significant difference in graft survival for the LRRN2 high/donor age ≥ 50 years group, but not in the other groups (Figure 4D, Table S8), suggesting that the expression of LRRN2 in elderly donors could serve as a biomarker for graft failure in recipients. The number of LRRN2 high/donors age < 50 years was a little bit small. Hence, we added another analysis to use the other donor age cutoff value, 40 or over 40 years, to validate the classification with LRRN2 expression and donor age.[31] The graft survival rate in LRRN2 high/donor age ≥ 40 years group was significantly poor in the analysis with 40-year cutoff value, similar to the analysis with 50-year cutoff value in Figure S5.

DISCUSSION

The relationships between aging and hepatic gene-expression profiles remain nebulous, as only a limited number of studies have examined the effect of aging on liver function. The incidence of adverse metabolic changes, such as gluconeogenic capacity deterioration, increased lipid accumulation, and enhanced liver lipotoxicity and steatosis, is high in the elderly population.[11,32] Additionally, the aging liver has decreased regeneration capacity, which must be considered when determining graft quality following partial LT.[11] Cellular senescence is a critical factor in liver regeneration. Aging livers have fewer mitochondria and increased hepatocyte size, which may promote polyploidy, an indicator of cellular senescence and stress response that impairs proliferation.[11] Moreover, Timchenko et al.[33] reported that the hepatic levels of the CCAAT/enhancer-binding proteinα-Brm-HDAC1 complex increase with aging and inhibit liver regeneration by repressing E2F-dependent promoters. Therefore, cell proliferation is suppressed in the aged liver, presenting a major challenge for LDLT when using a small graft.

Our microarray study using a macaque model comparatively assessed the expression of previously reported senescence-associated genes (Figure 1B), including *CDKN1A* (encoding p21) and *CDKN2A* (encoding p16), which are reportedly negatively correlated with liver regeneration after resection.[14,26] In our samples, the expression levels of these genes, including *CDKN1A* and *CDKN2A*, were similar between the groups or increased with age. Furthermore, *SIRT1* expression was diminished, which also has been reported in rodent model aged liver grafts.[27] Collectively, these findings confirmed the suitability of cynomolgus macaques as an aging model for research regarding liver transplantation.

In small animals, including mice, several reports have examined liver genetic changes during aging.[15,34] Although Nicholas et al.[15] reported that the number of hepatic DEGs increased significantly with age, we did not observe this effect in aging macaques. However, multiple subpopulations were detected in our elderly group of cynomolgus macaques; one such "subpopulation" exhibited a genetic profile similar to that of the young group, while the other had a genetic profile that differed entirely from the young group. Thus, we hypothesized that the elderly subgroup of cynomolgus macaques may exhibit changes in the hepatic expression of specific genes and that these genes can determine the graft quality in elderly and young macaques.

By comparing the young and elderly groups of macaques, we found 467 genes that were significantly up-regulated in the elderly group. Using this method, five of the six genes we identified predicted graft failure with a high probability. Moreover, this division enabled us to identify markers whose expression are uniformly increased in older individuals, such as *CDKN1A* and *CDKN2A*. We found that graft failure was significantly more common in the group exhibiting up-regulated expression of the six candidate genes. Limited studies have examined the correlation between the expression of these six genes and LT. *ATP1A1*, which regulates mitochondrial metabolism, is involved in the progression of nonalcoholic fatty liver disease or nonalcoholic steatohepatitis before and after LT.[35,36] LTBP4, which accumulates in the extracellular matrix of patients with stage F3 liver fibrosis, is reportedly involved in the activation of transforming growth factor beta (TGF-β) and induction of liver cirrhosis, while suppression of LTBP4 or FOSB promotes cell proliferation.[37,38] In neuroblastoma, LRRN2 was correlated with cell proliferation in the JNK pathway.[39] This suggested that cell proliferation may be regulated by various factors in the liver, and further studies are needed to determine whether suppression of LTBP4 or FOSB may promote hepatocyte proliferation in grafts and improve graft survival in LDLT. Consistently, microarray data demonstrated the up-regulation of *TGFB1* in the elderly (Figure 1B). Liver steatohepatitis and fibrosis affect the chronic course of LT. Collectively, these observations suggest that these six genes may be involved in the acute stage of liver damage through unknown mechanisms. However, due to the small number of candidates, we were not able to identify the pathways or mechanisms involved in graft failure.

Multivariable analysis revealed that the donor age, GV/SLV or GRWR, MELD score, and absence of splenectomy were predictive factors for graft failure, which was consistent with the results of previous studies.[5,8,40] Several recent studies have shown that donor age does not affect graft viability.[41,42] However, these studies

included only patients who underwent LDLT with right lobe grafts, which have large volumes. Previously, we reported that donor age was a risk factor for graft failure when the graft volume was small or when the clinical condition of the recipient was poor[8] due to the reduced proliferative capacity of aged liver.[40] The quality of grafts is critical when LDLT is performed with smaller grafts, such as the left lobe. Left lobe grafts are safer for the donor and should be used if it is sufficient for recipients.

In addition to the predictive factors that have been reported, high expression of *LRRN2* in the donor liver was a predictive factor identified via multivariable analysis. Therefore, *LRRN2* should be considered as an objective predictor of graft failure. Additionally, the combination of *LRRN2* expression and donor age correlated with early graft failure. This is an important clue for determining the mechanism of deterioration in graft quality and identifying additional LDLT graft quality markers. Expression of *LRRN2*, which is a member of the leucine-rich repeat superfamily and is involved in signal transduction and cell adhesion, was first reported to be up-regulated in patients with malignant gliomas.[43] The function of LRRN2 in the liver has not been previously reported. Here we showed that LRRN2 is expressed in the liver of donors at the mRNA and protein level. In addition, we investigated the correlation with *LRRN2* expression and several genes, such as aging-related genes (*CDKN2A* and *CDKN1A*), fibrogenesis-related genes (matrix metalloproteinase [*MMP*]*-2* and *MMP-9*), and liver regeneration–related genes (*MET* and *IL-6R*), in microarray data of cynomolgus macaques (Figure S6). Surprisingly, LRRN2 expression is strongly correlated with *MMP-9* expression. It is reported that MMP-9 was correlated with ischemia/reperfusion injury,[44] although MMP-9 is virtually absent in naive liver.[45] In the MMP-$9^{-/-}$-deficient steatotic mouse model, the ischemia/reperfusion injury was reduced.[45] Hence, the relationship among LRRN2 expression, MMP-9 expression, and ischemia/reperfusion injury in human specimens should be verified in the future. Further experimental studies are needed to better understand the mechanisms underlying LRRN2-mediated correlation with graft quality.

Although our findings suggest that *LRRN2* expression can predict graft failure, the clinical use of LRRN2 as a biomarker is associated with certain limitations. Similar to markers such as CD68, a liver biopsy is required to evaluate *LRRN2* expression. Previous studies have shown that deaths due to liver biopsy during LT were limited to patients with malignant disease and liver cirrhosis. The percutaneous liver biopsy mortality rate was approximately 0.1%,[46] with bleeding being the main complication.[46] The mortality and morbidity rates in donors with normal liver function are expected to be lower than those in patients with liver disease. However, there is still a need to develop minimally invasive techniques, such as liquid biopsy. The aging state of blood immune cells is closely correlated with the aging of solid organs.[47] The elder 2-year-old mouse had about 10 times higher senescence markers in blood CD3-positive cells, such as p16 and P21, compared with the young 8–10-month-old mouse. On the other hand, liver p16 and p21 expression increased 10-fold in the elder 2-year-old mouse compared with the young 8–10-month-old mouse. In addition, oxidative DNA level in liver also increased about 15-fold. Induction of cellular stress was reported to increase the LRRN2 expression in neuroblastoma cells.[39] These reports suggest that the expression of LRRN2 in the liver may be related to the state of immune cells in the blood. Hence, we are planning the following research to examine factors to predict graft aging and quality from donor peripheral blood mononuclear cells. This might enable donors to investigate graft status less invasively. Clinically important genes must be reconsidered with other factors, such as steatosis and CIT, which affect graft quality.[9–11] The six candidate genes analyzed in this study may also affect the quality of deceased donor grafts. However, ATP1A1 has been reported to be involved in fatty transformation of the liver before and after transplantation and may markedly affect graft quality in deceased donor LT.[35,36] Thus, our study outcomes may be applicable to deceased donor LT as well.

The localization of LRRN2 expression in liver is unclear by quantitative real-time PCR. Hence, we performed IHC to determine the localization of LRRN2 expression and found that only hepatocytes expressed LRRN2 in Figure 4A. This localization of LRRN2 expression was consistent with the results of the Protein Human Atlas. However, the expression of intrahepatic cells other than hepatocytes may be masked by hepatocytes. The relationship between intrahepatic cells other than hepatocytes and LRRN2 expression should be verified in the following study.

This study has some limitations. First, it was a retrospective, single-center study, so a multi-institutional study must be performed in the future to adjust the cutoff value of LRRN2 expression. We have shown the distribution and summary of respective gene expression in donor liver in Figure S7 for a subsequent study. Second, we used cynomolgus macaques, which are one of the best model animals for human aging; however, the aging process of cynomolgus macaques still differs from that of humans. Therefore, a larger number of human donor samples must be analyzed to overcome the difference in human donor background factors and identify new markers for predicting graft failure. Third, all of our elderly cynomolgus macaques were females; however, the human hepatic expression levels of six genes identified in this study were not markedly different between males and females. For these reasons, the results of this study should be generalized with caution.

In conclusion, donor hepatic *LRRN2* expression may be a valuable marker for evaluating graft quality in LDLT.

AUTHOR CONTRIBUTIONS

Study concept: Takahiro Tomiyama, Tomoharu Yoshizumi, Shokichi Takahama, and Takuya Yamamoto. *Data collection*: Takahiro Tomiyama, Tomoharu Yoshizumi, NG, Shinji Itoh, and Takeo Toshima. *Statistical analysis*: Takahiro Tomiyama, Shokichi Takahama, and Mototsugu Shimokawa. *Manuscript draft*: Takahiro Tomiyama and Shokichi Takahama. *Critical revision of the manuscript*: Tomoharu Yoshizumi, T. Yamamoto, and Masaki Mori. All authors approved the final version of the manuscript.[48–50]

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CONFLICT OF INTEREST

Nothing to report.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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