Katsuro Koike (Department of Gene Research, The Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan) (Shirakata and Koike, 2003). The HA-tagged JNK1, JNK2, and c-Jun expression plasmids were provided by Michael Karin (Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, School of Medicine, University of California, San Diego, USA). The GST-fused recombinant HBx plasmid pGEX-HBx was described previously (Tanaka *et al.*, 2004).

The plasmid pAP-1-Luc, containing the *Photinus pyralis* (firefly) luciferase reporter gene driven by a basic promoter element (TATA box) plus seven repeats of the binding site for AP-1 (TGACTAA), and pRL-TK, *Renilla reniformis* (sea pansy) luciferase driven by the herpes simplex virus thymidine kinase promoter, were purchased from Stratagene (La Jolla, CA, USA) and Promega (Madison, WI, USA) respectively.

The short hairpin RNA expression plasmid for stable knockdown under the control of U6 promoter, pcPUR + U6icassette, was provided by Makoto Miyagishi and Kazunari Taira (Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, Tokyo, Japan) (Miyagishi and Taira, 2002; Jazag *et al.*, 2005). The sequence for targeting Jab1 was 5′-CATGCAGGAAGCTCAGAGTAT-3′ and pcPUR + U6-Jab1i was constructed.

All constructs were verified by DNA sequencing.

MEF purification

MEF purification was carried out as described previously (Ichimura *et al.*, 2005). Briefly, 293T cells in eight 10 cm dishes (approximately 8×10^7 cells) were transfected with 16 μg of plasmid (pcDNA3-MEF-HBx or pcDNA3-MEF). After 48 h, the cells were harvested with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (w/v) glycerol, 100 mM NaF, 10 mM EGTA, 1 mM Na_3VO_4, 5 μM $ZnCl_2$, 1% (w/v) Triton X-100, and the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany)) and centrifuged at 15 000 *g* for 20 min at 4°C. The supernatant was incubated with anti-myc Sepharose for 90 min at 4°C and washed five times with TNTG buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (w/v) glycerol, 0.1% (w/v) Triton X-100), twice with buffer A (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (w/v) Triton X-100), and finally with TNT buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (w/v) Triton X-100]. The beads were then mixed with 15 U tobacco etch virus (TEV) protease (Invitrogen, Carlsbad, CA, USA) in TNT buffer at room temperature for 1 h to release the protein complex from the beads. The protein complex was incubated with M2-agarose beads for the second immunoprecipitation at room temperature for 1 h and washed with buffer A three times. The beads were subsequently eluted with flag peptide in buffer A (80 μg/ml). The eluted proteins were concentrated, separated by 10/20% SDS–PAGE, and visualized by silver staining, according to a modified protocol (Imamura *et al.*, 2004).

Tandem mass spectrometry

Proteins were identified as previously described (Natsume *et al.*, 2002; Ichimura *et al.*, 2005). Protein bands were excised from the gel plate and digested with trypsin, and the resultant peptide fragments were analysed using the direct nano-flow LC-MS/MS system, equipped with an electrospray interface reversed-phase column, a nano-flow gradient device, and a high-resolution quadrupole time-of-flight hybrid mass spectrometer (Q-TOF2; Micromass, Manchester, UK). All of the MS/MS spectra were searched against the non-redundant protein sequence data base maintained at the National Center for Biotechnology Information in order to identify proteins, using the Mascot program (Matrixscience, London, UK). The MS/MS signal assignments were confirmed manually.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described (Kanai *et al.*, 2000). Cells were lysed in lysis buffer, and cellular proteins were immunoprecipitated with antibody for 1 h at 4°C and with protein G-Sepharose (Zymed, San Francisco, CA, USA) overnight at 4°C. After the beads were washed with TNE buffer, the bound proteins were eluted by boiling in SDS sample buffer, separated by 10% SDS–PAGE, and electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF) Hybond-P (Amersham Biosciences). The membrane was probed with primary antibody (anti-flag, anti-HA, anti-Jab1, anti-phospho-c-Jun (Ser-63) II antibody, or anti-phospho-SAPK/JNK (Thr-183/Tyr-185) antibody at 1:1000 dilution) and with secondary antibody (anti-mouse antibody or anti-rabbit antibody conjugated with HRP at 1:1000 dilution). The HRP signal was detected with enhanced chemiluminescence ECL-plus (Amersham Biosciences).

Expression and purification of recombinant proteins of HBx and in vitro *pull-down assay*

Bead-immobilized GST fusion proteins were prepared as previously described (Tanaka *et al.*, 2004). In brief, *Escherichia coli* BL21(DE3) (Stratagene) transformed with pGEX-HBx or pGEX-4T-1 were induced with 1 mM IPTG at 37°C for 3 h, pelleted, and lysed in phosphate-buffered saline (PBS) containing 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 100 nM pepstatin, and 1% Triton X-100. The lysates were sonicated and clarified by centrifugation (8000 *g* for 10 min at 4°C). The fusion proteins were purified from the cleared bacterial lysates using glutathione-Sepharose 4B (Amersham Biosciences).

The *in vitro* transcription and translation of Jab1 were performed using the TNT T7 Coupled Reticulocyte Lysate systems (Promega). Translated Jab1 was incubated with bead-immobilized GST-HBx or GST control beads in lysis buffer overnight at 4°C. The beads were washed five times with TNTG buffer, and bound proteins were eluted, separated by 10% SDS–PAGE, and probed with streptavidin conjugated with HRP.

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out as previously described (Tateishi *et al.*, 2001). HeLa cells were seeded in a slide flask (Nalge Nunc, Naperville, IL, USA) at a density of 3×10^4 cells/cm^2 and transfected with the expression vectors 24 h later. After another 24 h, the cells were fixed with 2% paraformaldehyde for 10 min at room temperature, rinsed with PBS, and permeabilized with 0.25% Triton X-100/PBS for 10 min. The cells were then rinsed with PBS and incubated with rabbit anti-Jab1 polyclonal antibody (1:100 dilution) for 1 h at room temperature, followed by incubation with Alexa Fluor 488 or 555 goat anti-rabbit IgG secondary antibody (1:100 dilution) for 1 h at room temperature. The nuclei of the cells were stained with propidium iodide (Sigma). The cells were mounted with DAKO Fluorescent mounting medium (DAKO, Carpinteria, CA, USA). Fluorescence images were obtained using a Leica TCS SL confocal laser scanning microscope (Leica Microsystems, Wetzler, Germany) and photographed using Leica confocal software (Leica Microsystems).

Establishment of Jab1 knockdown HeLa cells
HeLa cells were transfected with pcPUR + U6-Jab1i or pcPUR + U6icassette, incubated with 1 μg/ml puromycin to select stably transfected cells and Jab1 knockdown cell pool and wild-type cell pool were established.

Luciferase assays
Luciferase assays were performed as described previously (Kato *et al*., 2000). Approximately 4×10^4 HeLa cells were plated into each well of a 12-well tissue culture plate (Iwaki Glass, Chiba, Japan) 24 h before transfection. The complexes, containing a total of 0.3 μg of plasmid (0.14 μg pAP-1-Luc, 0.01 μg pRL-TK, and 0.15 μg of other plasmids), were transiently transfected into HeLa cells. Empty vector, pCMV, was added to each transfection to adjust the total amount of DNA to 0.3 μg. The cells were harvested 24 h after transfection and assayed for luciferase activity using the PicaGene dual seapansy system (Toyo Ink, Tokyo, Japan). Firefly luciferase activity was measured as relative light units using a luminometer (Lumat LB9507, EG and G Berthold, Bad Wildbad, Germany). pRL-TK was used to standardize the efficiency of transfection. Independent experiments were performed at least three times.

Statistics
All data are shown as the mean ± s.d. The results were analyzed using Student's *t*-test. *P*-values <0.05 were considered significant.

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ONCOGENOMICS

Absence of *PIK3CA* hotspot mutations in hepatocellular carcinoma in Japanese patients

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A recent study revealed that the p110α (*PIK3CA*), catalytic subunit of phosphatidylinositol 3-kinase (PI3K), is somatically mutated in many types of cancer. For example, *PIK3CA* is mutated in an estimated 35.6% of hepatocellular carcinoma (HCC) cases. To measure the frequency of *PIK3CA* hotspot mutations in Japanese HCC patients, exons 9 and 20 of the *PIK3CA* gene were sequenced in 47 clinical HCC samples. Contrary to expectations, no hotspot mutations were found any of the HCC samples. In addition, we found abnormally migrating waves near the end of exon 9 in the PCR chromatograms from 13 of the 47 samples. PCR amplification and subsequent cloning and sequencing revealed that these chromatograms contained two distinct sequences, the wild-type p110α sequence and a different sequence found on human chromosome 22q11.2, the Cat Eye Syndrome region, which contains a putative pseudogene of *PIK3CA*. These abnormally migrating waves were also found in noncancerous liver tissue, indicating that this was not a result of HCC-associated mutations. Therefore, it is likely that the percentage of hotspot mutations in the *PIK3CA* gene of Japanese HCC patients is lower than was previously reported.
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Phosphatidylinositol 3-kinase (PI3K) is a family of lipid kinases that regulate the signaling pathways involved in cell survival and proliferation (Cantley, 2002). The aberrant activation of this pathway has been reported in many types of cancers, including colorectal carcinoma, ovarian carcinoma, and breast carcinoma (Vivanco and Sawyers, 2002). PI3Ks are expressed as heterodimers of catalytic and regulatory subunits. The catalytic subunits are encoded by three genes (α, β, γ), of which the p110α subunit (*PIK3CA*) was reported to be overexpressed in ovarian carcinomas and was thus implicated as an oncogene (Shayesteh *et al.*, 1999). Recently, Samuels *et al.* (2004) sequenced the coding exons of *PIK3CA* and showed that this gene was somatically mutated in a high frequency of colorectal carcinomas. They reported that the *PIK3CA* gene was mutated in 74 of 234 (32%) colorectal carcinomas, but in only two of 76 (3%) premalignant colorectal tumors, suggesting that these mutations arise late in tumorigenesis. Moreover, they found *PIK3CA* mutations in 27% of glioblastomas, 25% of gastric carcinomas, 8% of breast carcinomas, and 4% of lung carcinomas, indicating that these mutations are prevalent in many types of cancer. This observation was confirmed by several other groups (Campbell *et al.*, 2004; Levine *et al.*, 2005; Li *et al.*, 2005; Velho *et al.*, 2005). The *PIK3CA* mutations were located mostly at hotspots within the helical domain (encoded by exon 9) and the kinase domain (encoded by exon 20), and they resulted in gain of function mutations that were implicated in the initiation of oncogenicity (Ikenoue *et al.*, 2005; Kang *et al.*, 2005; Samuels *et al.*, 2005).

The *PIK3CA* gene was also reported to be highly mutated in approximately 35.6% of hepatocellular carcinoma (HCC) cases (Lee *et al.*, 2005). The most frequent *PIK3CA* mutations in HCC include a substitution mutation in the helical domain (A1634C) and an insertion mutation in the kinase domain (3204_3205 insA). The substitution mutation results in an amino acid change at the same residue (aa 545) in the p110α protein as that affected by a hotspot mutation reported in colorectal carcinomas. In HCC, the A1634C mutation results in a Glu (E) to Ala (A) change, thus replacing a charged (acidic) residue with a neutral residue, whereas, in colorectal carcinoma, the G1633C mutation changes a Glu (E) to Lys (K), thus replacing the charged (acidic) residue with a basic residue. A novel insertion mutation, first reported by them, changes the C-terminal Asn (N) of the p110α protein to Lys (K) and adds three amino acids, resulting in a more basic sequence, Lys-Leu-Lys-Arg (KLKR). Although functional analyses have yet to be performed, these mutations might cause the aberrant activation of PI3K and of the downstream kinase Akt, as seen in HCC.

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To investigate the frequency of *PIK3CA* mutations in Japanese patients with HCC, we sequenced the two hotspot-containing exons (9 and 20) in 47 HCC samples. The male to female ratio of the patients was 38:9, and their ages ranged from 28 to 81 years, with an average age of 62 years. In all, 10 patients had HCC associated with hepatitis B virus infection, 30 patients had HCC associated with hepatitis C virus infection, and seven patients had HCC not associated with hepatitis virus infection. The tumor sizes ranged from 1.2 to 10 cm (average 4.7 cm). This study was performed with appropriate Institutional Review Board approval.

Genomic DNA was extracted from tumors and normal tissues obtained from frozen surgical or biopsy specimens. The primers used for PCR amplification of *PIK3CA* exons and for sequencing were described previously (Samuels *et al.*, 2004). PCR amplification was performed using a high-fidelity PCR master mix (Roche, Mannheim, Germany), and reaction conditions were 30 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Direct sequencing was performed using the ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA).

In these experiments, no mutations were detected in exon 9 or 20 in any of the HCC samples. However, in 13 of 47 clinical samples, we noted that the chromatograms resulting from the exon 9 PCR reaction contained abnormally migrating waves near the end of exon, as shown in Figure 1a. PCR products from noncancerous liver tissue also had these abnormally migrating waves, indicating that this was not a result of HCC-associated mutations. PCR amplification and subsequent cloning and sequencing of the PCR products revealed that the sequence chromatograms contained two distinct sequences (Figure 1b and c), the wild-type p110α sequence (GAGCAGGAGAAAGATTTTCTATGGAGTCACAG) and a similar but distinct sequence (GCGCAGGAGAA AGATTTTCTATGGACCACAG; base changes are underlined). A BLAST search showed that the latter sequence is found on human chromosome 22q11.2, the Cat Eye Syndrome (CES) region. CES is a hereditary disease characterized by ocular colobomata, anal atresia, congenital heart defects, and mental retardation. Patients with CES have a partial tetrasomy of the region that spans the p-arm and part of 22q11 (McDermid and Morrow, 2002). The 22q11 region contains partial sequences that are highly homologous to exons 9, 10, 11, 12, and 13 of the *PIK3CA* gene (98, 74, 98, 100 and 98%, respectively), thus this region is considered to be a putative pseudogene that arose as a result of gene duplication. This pseudogene does not seem to be transcribed, as an NCBI BLAST search revealed no expresses sequence tags (ESTs) with the same sequence. The primer for exon 9 anneals to both the *PIK3CA* and the pseudogene sequences, indicating that these primers were not specific for *PIK3CA*. The CES region sequence resembles a previously reported mutation in exon 9, but we did not consider this as a mutation because it reflects the sequence of the putative pseudogene rather than a mutated wild-type sequence. Similar findings have been reported by other groups (Or *et al.*, 2005; Saal *et al.*, 2005).

To confirm the hypothesis that the A1634C sequence change was a result of the amplification of the pseudogene and not a mutation, we designed a new reverse primer set specific for the amino-terminal region

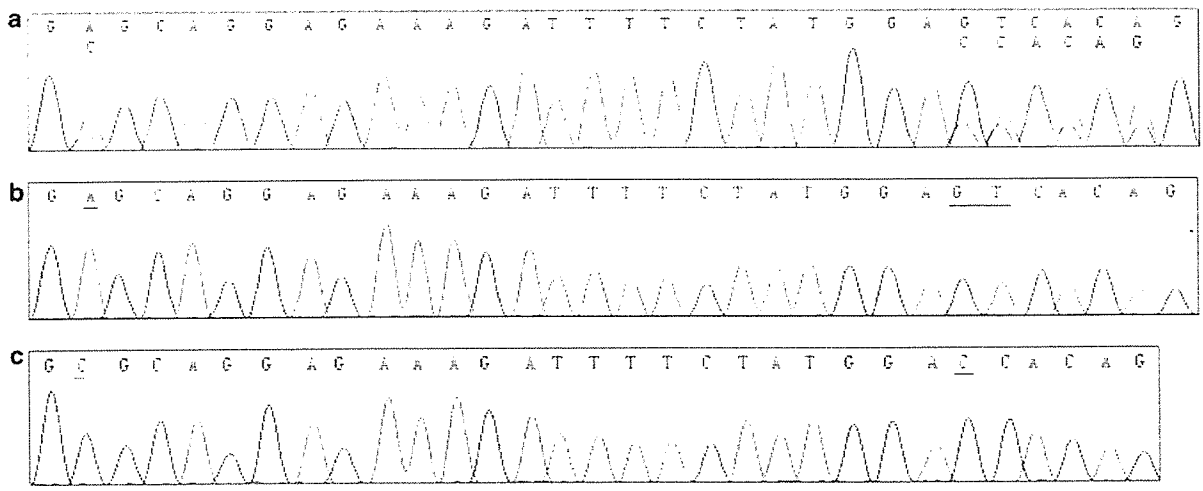

Figure 1 Sequence analysis of exon 9 of the *PIK3CA* gene in human HCC. (**a**) Sequence chromatogram of exon 9, which contained abnormally migrating waves near the end of exon. Genomic DNA was extracted from tumors and normal tissues obtained from frozen surgical or biopsy specimens. The primers used for amplification of exons and for sequencing were described previously (Samuels *et al.*, 2004). PCR amplification was performed using high-fidelity PCR master mix (Roche, Mannheim, Germany), and reaction conditions were 30 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Direct sequence was performed using the ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA). (**b** and **c**) Distinct sequences were obtained from the PCR amplification of exon 9. The cloning of the amplified PCR products was performed with TOPO TA cloning (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequent sequencing revealed two different sequences: 1633-GAGCAGGAGAAAGATTTTC TATGGAGTCACAG-1664 (**b**) and 1633-GCGCAGGAGAAAGATTTTCTATGGACCACAG-1663. (**c**). Underlining indicates base changes between the two sequences. Representative sequence chromatograms are shown.

of exon 10 of the *PIK3CA* gene (forward primer, 5′-GATTGGTTCTTTCCTGTCTCTG-3′; reverse primer; 5′-GTAGAATTTCGGGGATAGTT-3′) and performed PCR and sequence analysis. This primer yielded an amplicon of approximately 1100 base pairs, the sequence of which matched that of the wild-type *PIK3CA* gene (Figure 1b) but not that of the pseudogene (Figure 1c).

Although our results do not suggest that mutations in *PIK3CA* do not occur, the percentage of mutations is likely to be lower than previously reported (Lee *et al.*, 2005). Whether the PI3K-Akt pathway is activated in HCC remains controversial. A recent study indicated that the phosphorylation of the downstream kinases mTOR and P70 S6 was observed in 15 and 45% of HCC cases, respectively, suggesting that the PI3K-AKT pathway is indeed activated in a subset of HCC cases (Sahin *et al.*, 2004). Our findings do not address whether *PIK3CA* mutations directly cause the activation of the PI3K-Akt pathway in HCC. The relationship between HCC and the PI3K-Akt pathway remains to be determined.

In conclusion, our results suggest that the percentage of hotspot mutations in the *PIK3CA* gene is lower among Japanese patients with HCC than was previously reported.

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Prediction of Recurrence of Hepatocellular Carcinoma After Curative Ablation Using Three Tumor Markers

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Three tumor markers for hepatocellular carcinoma (HCC) are available in daily practice in Japan: alpha-fetoprotein (AFP), des-gamma-carboxy prothrombin (DCP), and lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3). To elucidate the predictability of these tumor markers on HCC recurrence after curative ablation, we enrolled 416 consecutive patients with naïve HCC who had been treated by percutaneous ablation at our department from July 1997 to December 2002. Tumor marker levels were determined immediately before and 2 months after the treatment. Complete ablation was defined on CT findings as nonenhancement in the entire lesion with a safety margin. Tumor recurrence was also defined as newly developed lesions on CT that showed hyperattenuation in the arterial phase with washout in the late phase. We assessed the predictability of recurrence via tumor markers in multivariate analysis, using proportional hazard regression after adjusting for other significant factors in univariate analysis. Until the end of follow-up, tumor recurrence was identified in 277 patients. Univariate analysis revealed the following factors to be significant for recurrence: platelet count; size and number of tumors; AFP, AFP-L3, and DCP preablation; and AFP and AFP-L3 postablation. Multivariate analysis indicated that AFP >100 ng/mL and AFP-L3 >15%, both pre- and postablation, were significant predictors. The positivity of AFP and AFP-L3 preablation that turned negative postablation was not significant. In conclusion, tumor markers pre- and post-ablation were significant predictors for HCC recurrence and can complement imaging modalities in the evaluation of treatment efficacy. (HEPATOLOGY 2006;44:1518-1527.)

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, and its incidence is increasing in the United States and elsewhere.[1,2] Current options for the treatment of this cancer consist of surgical resection, orthotropic liver transplantation, transcatheter arterial embolization, and percutaneous ablation therapy. Although surgical resection is usually the first choice for treatment,[3,4] it is frequently contraindicated by underlying chronic liver disease based on hepatitis B or C virus infection.[5,6] Orthotopic liver transplantation is a strategy that can treat both cancer and liver dysfunction, and indeed has shown an excellent survival rate in patients at an early stage of the cancer (*e.g.*, single nodule measuring ≤5 cm in diameter or fewer than three nodules measuring ≤3 cm in diameter).[7,8] However, in countries such as Japan, where cadaveric donor organs are scarce, application of liver transplantation is limited. Percutaneous ablative methods—including percutaneous ethanol injection therapy, percutaneous microwave coagulation therapy, and radiofrequency ablation—can achieve high local cure without deteriorating background liver function[9-15] and have played an important role in the treatment of HCC.

Alpha-fetoprotein (AFP) has served as a diagnostic test for HCC since the 1970s, when most patients with HCC were diagnosed at an advanced stage with clinical symptoms.[16] Presently, small (*e.g.*, ≤3 cm) HCCs can often be detected due to advances that have been made in the various imaging techniques.[17,18] They do not usually secrete a diagnostic level of AFP,[19] rendering the role of AFP as a diagnostic test less significant.[20] On the other hand, AFP is known to be an important predictor of prognosis,[21-23] recurrence in particular. To date, several tumor markers have been proposed as a complement or substi-

Abbreviations: HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

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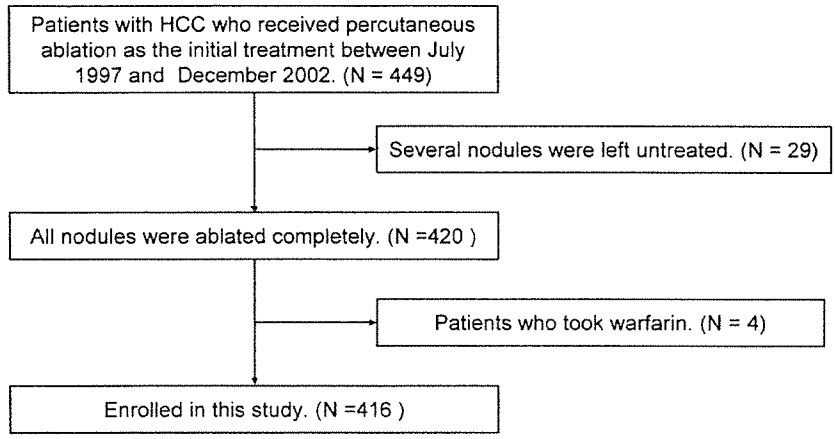

Fig. 1. Schematic flowchart of enrolled patients. Abbreviation: HCC, hepatocellular carcinoma.

tute for AFP in HCC diagnosis, such as des-gamma-carboxy prothrombin (DCP)[24,25] and lens culinaris agglutinin-reactive fraction of AFP (AFP-L3),[26,27] but no studies have compared the predictability of these tumor markers simultaneously on HCC recurrence after curative treatment. The aim of this study was to elucidate the predictability of these tumor markers on the recurrence after a curative ablation of HCC.

Patients and Methods

Patients. We determined the tumor markers AFP, DCP, and AFP-L3 in each patient with HCC admitted to the Department of Gastroenterology at the University of Tokyo Hospital since July 1997. A total of 449 patients received percutaneous ablation as the initial treatment for HCC from July 1997 to December 2002. Among them, 416 were enrolled in this study, excluding 29 in whom percutaneous ablation was intended to reduce tumor burden because of multinodularity with some nodules left unablated, and 4 who took oral warfarin, a DCP-inducing agent (Fig. 1). Informed written consent was obtained from each patient before treatment.

Diagnosis of HCC. HCC was diagnosed using dynamic CT, considering hyperattenuation in the arterial phase with washout in the late phase as definite HCC.[28] Most nodules were also confirmed histopathologically with ultrasound-guided biopsy. The pathological grade was based on Edmondson-Steiner criteria.[29]

Treatment and Follow-up. Inclusion criteria for percutaneous ablation were as follows: total bilirubin concentration <3 mg/dL; platelet count no less than $50 \times 10^3/mm^3$; and prothrombin activity no less than 50%. Patients with portal vein tumor thrombosis, refractory ascites, or extrahepatic metastasis were excluded. In general, we performed percutaneous ablation on patients with three or fewer lesions, all of which were ≤3 cm in diameter. However, we also performed ablation on patients with more than three lesions or lesions >3 cm in diameter if the procedure could be assumed to be clinically effective. The procedure has been meticulously described elsewhere.[30]

After several sessions of percutaneous ablation, dynamic CT was performed 1 to 3 days after the last session with a slice thickness of 5 mm to evaluate treatment efficacy. The interval between the initiation of contrast material infusion and CT image recording was 30 and 120 s for single-detector row spiral CT (Highspeed Advantage; GE Medical Systems, Milwaukee, WI) and 25, 40, and 120 s for multidetector-row CT (LightSpeed QX/I; GE Medical Systems). The images were presented after axial reconstruction with a slice thickness of 5 mm. Complete ablation was defined on CT findings as nonenhancement in the entire lesion with a safety margin in the surrounding liver parenchyma. Patients received additional sessions of ablation until complete ablation was confirmed in each nodule. We also assessed the changes in three tumor markers before and after curative ablation. The levels obtained before therapy and 2 months after the confirmation of complete ablation were adopted for analysis.

The follow-up consisted of monthly blood tests and monitoring of tumor markers at the outpatient clinic, and ultrasonography and dynamic CT scan were performed every 4 months. Tumor recurrence was defined according to the same criteria applied to the initial HCC. Intrahepatic HCC recurrence was classified as either recurrence at a site distant from the primary tumor or adjacent to the treated site (local tumor progression).[31] The tumor markers were assessed also at the diagnosis of recurrence.

Analysis of Recurrence. Time to recurrence was defined as the interval between the first ablation and the detection of tumor recurrence, death without recurrence, or the last examination until December 31, 2004, whichever came first. Because tumor recurrence and death without recurrence are "competing risks," we used cumulative incidence estimation with competing risks described by Gray.[32]

Statistical Analysis. We first plotted the cumulative rate of HCC recurrence and that of death without recurrence stratified by the following variables obtained at the time of initial ablation therapy: age, sex, tumor size, number of nodules, pathological grade, Child-Pugh class, alanine aminotransferase level, platelet count, three tumor markers, positivity for viral markers (hepatitis B surface antigen and anti–hepatitis C antibody), and alcohol consumption. Polychotomous categorical data were represented by corresponding binary dummy variables. Continuous variables were transformed into ordinal categories. The predictability of each tumor marker before and after ablation was assessed with a multivariate proportional hazard regression model described by Fine and Gray and adjusted by factors shown to be significant in the univariate analysis.[33] The differences with a P value of less than .05 were considered statistically significant. All statistical analyses were performed with S-plus 2000 software (MathSoft Inc., Seattle, WA).

Results

Patient Profiles. The baseline characteristics of the patients (276 males and 140 females) are shown in Table 1. The median age was 67 years, with the 25th and 75th percentiles being 62 and 71 years. Most (80.8%) were HCV-positive. The mean size and number of nodules were 2.7 cm and 1.7, respectively. The mean number of treatment sessions required for complete ablation was 7.7, 2.0, and 2.0 for percutaneous ethanol injection therapy, percutaneous microwave coagulation therapy, and radiofrequency ablation, respectively. Until the end of the follow-up, tumor recurrence was identified in 277 patients. Of these, 248 had intrahepatic recurrence distant from the primary site of ablation, 19 had local tumor progression, 7 had distant recurrence and local tumor progression simultaneously, and 3 had extrahepatic recurrence. Forty patients died with no sign of recurrence. Only 5 patients were lost to follow-up. The cumulative probability of overall recurrence was 18.6%, 44.7%, 59.0%, 67.2%, and 72.0% at 1, 2, 3, 4, and 5 years, respectively. The cumulative probability of local tumor progression was 2.2%, 5.1%, 6.1%, 6.5%, and 6.5% at 1, 2, 3, 4, and 5 years, respectively. The cumulative probability of death without recurrence was 2.9%, 5.8%, 8.2%, 9.6%, and 10.6% at 1, 2, 3, 4, and 5 years, respectively.

Table 1. Baseline Characteristics of Patients (N = 416)

Variable	n (%)
Age*	67 (62-71)
Male	276 (66.3)
Viral infection	
HBsAg-positive	49 (11.8)
Anti-HCV-positive	336 (80.8)
Both positive	7 (1.7)
Both negative	38 (9.1)
Drinking >80 g/d	71 (17.1)
Severity of background liver disease	
Child-Pugh classification	
Class A	301 (72.3)
Class B	111 (26.7)
Class C	4 (0.96)
AST (IU/L)*	57 (41-81)
ALT (IU/L)*	54 (34-80)
Platelet count ($\times 10^3/mm^3$)*	103 (76-140)
Tumor characteristics	
Size	
≤2 cm	140 (33.7)
2.1-3 cm	161 (38.7)
3.1-5 cm	94 (22.6)
>5 cm	21 (5.0)
Number of nodules	
Single	249 (53.3)
2-3	142 (33.5)
>3	25 (13.2)
Edmondson grade (N/A, n = 31)	
Grade 1	90 (21.6)
Grade 2	260 (62.5)
Grades 3-4	35 (8.4)
AFP	
≤100 ng/mL	319 (76.7)
101-400 ng/mL	66 (15.9)
>400 ng/mL	31 (7.5)
DCP (mAU/mL)	
≤100	348 (83.6)
101-400	45 (10.8)
>400	23 (5.5)
AFP-L3 (%)	
≤15	359 (86.2)
15.1-40	24 (5.8)
>40	33 (7.9)
Treatment modality	
PEIT	100 (24.0)
PMCT	22 (5.2)
RFA	294 (70.7)

Abbreviations: HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; PEIT, percutaneous ethanol injection therapy; PMCT, percutaneous microwave coagulation therapy; RFA, radiofrequency ablation.

*Expressed as median (25th-7th percentiles).

Tumor Markers Before and After Ablation. We plotted the value of each tumor maker immediately before ablation against the value 2 months after ablation (Fig. 2).

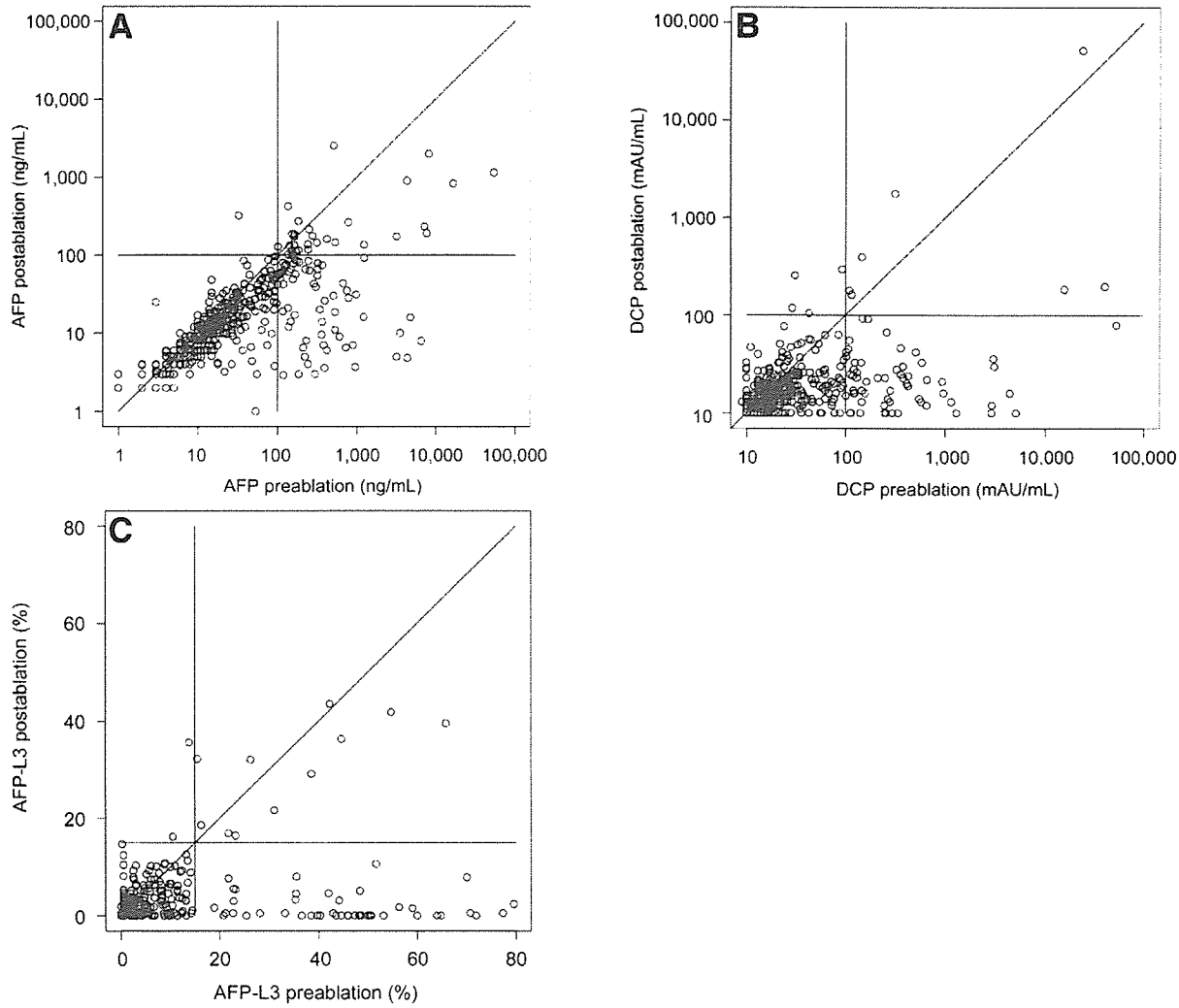

Fig. 2. Scatter plots of (A) alpha-fetoprotein, (B) des-gamma-carboxy prothrombin, and (C) lens culinaris agglutinin-reactive fraction of AFP pre- and postablation. AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

With cutoff values of 100 ng/mLfor AFP, 100 mAU/mL for DCP, and 15% for AFP-L3, the sensitivities were 23.3%, 16.3%, and 13.7%, respectively. At least one marker was positive in 151 (36.3%) patients. After curative ablation, the value decreased below the cutoff level in 67 of 97 (69.1%), 61 of 68 (89.7%), and 46 of 57 (80.7%) patients for AFP, DCP, and AFP-L3, respectively. In Fig. 2A, dots were clustered near the diagonal line when AFP before ablation was <100 ng/mL, indicating that the changes through ablation were minimal. In those cases, AFP before ablation seems to have been produced mainly in the background liver. Although the cutoff value of 20 ng/mL is often adopted for AFP as a HCC biomarker, this value may be too low in the evaluation of efficacy of HCC treatment. In contrast, the ordinary cutoff values of 100 mAU/mL for DCP and 15% for AFP-L3 also appear to be suitable for the evaluation of treatment (Fig. 2B-C).

Predicting Factor for Recurrence. Univariate analysis identified the following variables as significant predictors for recurrence: platelet count, size, and number of tumor nodules, AFP pre- and postablation ($P < .001$ and $P < .001$), and AFP-L3 pre- and postablation ($P = .044$ and $P < .001$) as significant predictors for recurrence (Table 2, Fig. 3). DCP preablation was also a significant predictor ($P = .020$) when a cutoff value of 100 mAU/mL was adopted. Child-Pugh classification was not significant for recurrence, whereas it strongly affected the probability of death without recurrence (Fig. 3A). Multivariate analysis with proportional hazard model revealed that AFP >100 ng/mL preablation and AFP-L3 >15% preablation were significant predictors of recurrence after

Table 2. Cumulative Probability of Recurrence and Death Without Recurrence After Curative Ablation

Variable (n)	Cumulative Probability of Recurrence (%)			Cumulative Probability of Death Without Recurrence (%)		
	1 Year	2 Years	*P*	1 Year	2 Years	*P*
Age ≤67 (214)	16.8	43.5	.13	2.8	4.7	.32
Age >67 (202)	20.4	45.9		3.0	7.0	
Male (276)	17.8	44.5	.64	3.2	6.9	.37
Female (140)	20.0	45.0		2.1	3.6	
HBsAg-negative (367)	18.6	45.2	.61	2.7	6.0	.36
HBsAg-positive (49)	18.4	40.8		4.1	4.1	
HCVAb-negative (80)	16.5	36.7	.082	5.0	5.0	.47
HCVAb-positive (336)	19.0	46.6		2.4	6.0	
Drinking ≤80 g/d (345)	18.3	43.7	.62	3.2	6.1	.66
Drinking >80 g/d (71)	19.7	49.3		1.4	4.2	
Child-Pugh A (301)	17.3	42.2	.45	1.0	2.3	<.001
Child-Pugh B/C (115)	21.9	51.1		7.9	15.0	
Platelet count						
≤100 × 10^3/mm^3 (198)	20.2	50.2	.030	3.0	8.1	.061
101-150 × 10^3/mm^3 (127)	18.9	43.5		3.9	4.7	
>150 × 10^3/mm^3 (91)	14.4	34.4		1.1	2.2	
ALT ≤80 IU/L (312)	18.0	43.8	.29	2.9	6.1	.81
ALT >80 IU/L (104)	20.2	47.4		2.9	4.8	
Size of tumor						
≤2.0 cm (140)	10.0	35.1	.009	0.7	4.3	.55
2.1-3.0 cm (161)	16.8	46.0		4.3	6.2	
3.1-5.0 cm (94)	27.7	53.5		4.3	8.6	
>5.0 cm (21)	50.0	60.0		0	0	
Single nodular (249)	16.5	38.0	<.001	2.8	6.1	.44
Multinodular (167)	21.6	54.7		3.0	5.4	
Edmondson grade 1 (90)	13.3	36.8	.18	3.3	3.3	.11
Edmondson grade ≥ 2 (295)	19.3	47.2		3.1	7.1	
AFP preablation						
≤100 ng/mL (319)	15.7	41.2	<.001	3.4	5.7	.82
101-400 ng/mL (66)	19.7	48.5		1.5	7.6	
>400 ng/mL (31)	46.7	73.3		0	3.3	
DCP preablation						
≤100 mAU/mL (348)	15.5	41.8	.099	2.6	5.5	.43
101-400 mAU/mL (45)	33.3	60.0		6.7	6.7	
>400 mAU/mL (23)	36.4	59.1		0	9.0	
AFP-L3 preablation						
≤15% (259)	17.0	42.6	.044	2.5	5.3	.62
15.1%-40% (24)	25.0	66.7		4.2	8.3	
>40% (33)	30.3	51.5		6.1	9.1	
AFP postablation						
≤100 ng/mL (385)	17.1	42.7	<.001	3.1	6.0	.80
101-400 ng/mL (25)	29.2	66.7		0	4.2	
>400 ng/mL (6)	66.7	83.3		0	0	
DCP postablation						
≤100 mAU/mL (405)	17.8	44.4	.35	2.5	5.2	.12
>100 mAU/mL (9)	44.4	44.4		22.2	33.3	
>400 mAU/mL (2)	50	NA		0	NA	
AFP-L3 postablation						
≤15% (403)	16.9	43.1	<.001	3.0	5.7	.57
15.1%-40% (11)	72.7	NA		0	NA	
>40% (2)	50.0	NA		0	NA	

Abbreviations: HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; ALT, alanine aminotransferase; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; NA, not available.

adjusting for other significant predictors—namely, platelet count and size and number of tumor nodules (Table 3). DCP >100 mAU/mL preablation showed marginal significance ($P = .069$).

In subgroup analysis, AFP >100 ng/mL preablation was not a significant predictor among patients in whom the value had decreased below 100 ng/mL after ablation. On the other hand AFP >100 ng/mL both pre- and

postablation was a significant risk for recurrence. Similarly, AFP-L3 >15% preablation was not significant in patients whose AFP-L3 became ≤15% after ablation, whereas AFP-L3 >15% pre- and postablation was the strongest predictor of recurrence (hazard ratio, 4.25; $P = .0096$). Those whose DCP value was > 100 mAU/mL before ablation but below 100 mAU/mL after ablation had a higher risk of recurrence than those whose DCP had been < 100 mAU/mL preablation.

Tumor Markers at Recurrence. Tumor markers were examined also at the diagnosis of recurrence in all 277 patients (Fig. 4). When cutoff values of 100 ng/mL, 100 mAU/mL, and 15% were applied for AFP, DCP, and AFP-L3, the sensitivities were 24.1%, 14.8%, and 22.4%, respectively. Among 71, 49, and 42 patients who were positive for AFP, DCP, and AFP-L3 before ablation, 43 (60.6%), 16 (32.7%), and 27 (64.3%) were positive for the corresponding marker also at the diagnosis of recurrence.

Table 3. Hazard Ratio of Tumor Markers as a Risk Factor for Tumor Recurrence Adjusted by Platelet Count, Size, and Number of Tumors

	Hazard Ratio (95% CI)	*P* Value
AFP		
≤100 ng/mL preablation	1	
>100 ng/mL preablation	1.45 (1.09-1.94)	.01
& ≤100 ng/mL postablation	1.23 (0.88-1.72)	.22
& >100 ng/mL postablation	2.31 (1.45-3.70)	<.001
DCP		
≤100 mAU/mL preablation	1	
>100 mAU/mL preablation	1.37 (0.98-1.93)	.069
& ≤100 mAU/mL postablation	1.44 (1.02-2.04)	.041
& >100 mAU/mL postablation	0.91 (0.27-3.13)	.89
AFP-L3		
≤15% preablation	1	
>15% preablation	1.52 (1.06-2.18)	.023
& ≤15% postablation	1.24 (0.85-1.81)	.26
& >15% postablation	4.25 (1.42-12.74)	.0096

Abbreviations: AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

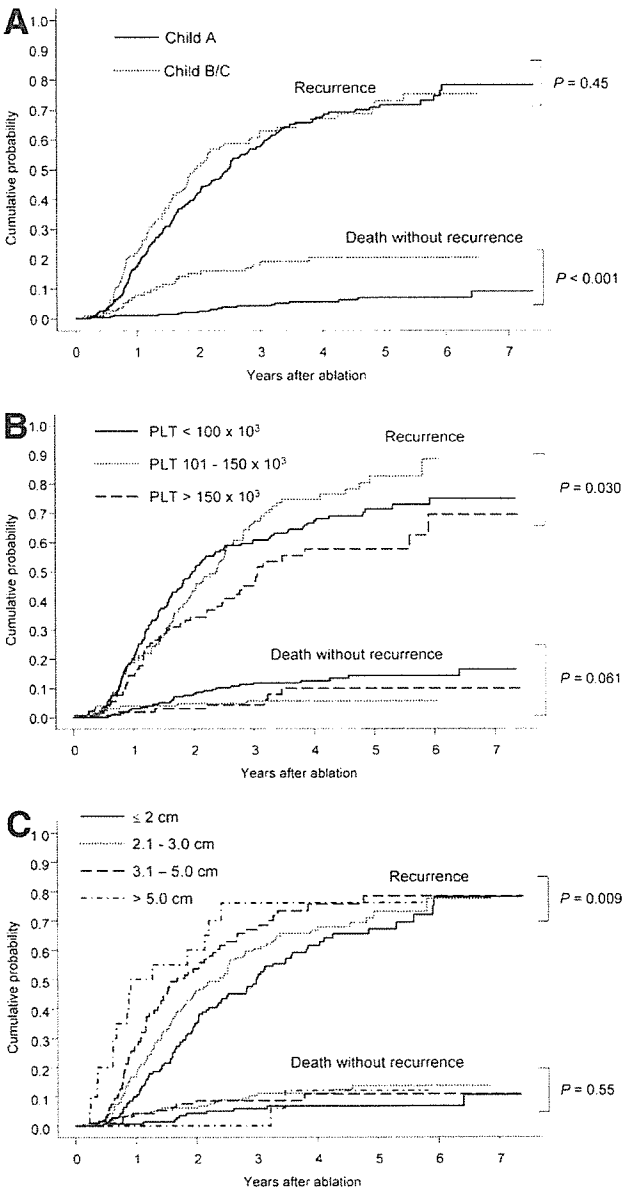

Fig. 3. (A) Cumulative probabilities of recurrence and death without recurrence divided by Child-Pugh class. Cumulative probabilities of recurrence at 1, 2, 3, 4, and 5 years were 17.2%, 42.2%, 57.6%, 67.4%, and 71.7% for class A, and 21.9%, 51.1%, 62.8%, 67.0%, and 73.0% for class B/C, respectively. Cumulative probabilities of death without recurrence were 1.0%, 2.3%, 4.1%, 5.5%, and 6.9% for class A, and 7.9%, 15.0%, 19.0%, 20.4%, and 20.4% for class B/C, respectively. (B) Cumulative probabilities of recurrence and death without recurrence divided by platelet count. Cumulative probabilities of recurrence at 1, 2, 3, 4, and 5 years were 20.2%, 50.2%, 60.8%, 66.6%, and 71.4% for platelet count $<100 \times 10^3/mm^3$; 18.9%, 43.4%, 66.2%, 74.6%, and 82.6% for platelet count 101-150 $\times 10^3/mm^3$; and 14.4%, 34.4%, 45.0%, 57.7%, and 57.7% for platelet count $>150 \times 10^3/mm^3$, respectively. Cumulative probabilities of death without recurrence at 1, 2, 3, 4, and 5 years were 3.0%, 8.1%, 11.8%, 12.5%, and 14.4% for platelet count $\leq 100 \times 10^3/mm^3$; 3.9%, 4.7%, 5.7%, 5.7%, and 5.7% for platelet count 101-150 $\times 10^3/mm^3$; and 1.1%, 2.2%, 3.4%, 9.2%, and 9.2% for platelet count $>150 \times 10^3/mm^3$, respectively. (C) Cumulative probabilities of recurrence and death without recurrence divided by tumor size. Cumulative probabilities of recurrence at 1, 2, 3, 4, and 5 years were 10.0%, 35.1%, 49.9%, 61.4%, and 67.0% for tumor size ≤2 cm; 16.8%, 46.0%, 60.3%, 66.6%, and 73.1% for tumor size 2.1-3.0 cm; 27.7%, 53.5%, 66.8%, 75.5%, and 78.2% for tumor size 3.1-5.0 cm; and 50%, 60%, 76%, 76%, and 76% for tumor size >5.0 cm, respectively. Cumulative probabilities of death without recurrence at 1, 2, 3, 4, and 5 years were 0.7%, 4.3%, 5.9%, 6.8%, and 6.8% for tumor size ≤2 cm; 4.3%, 6.2%, 11.0%, 11.0%, and 13.7% for tumor size 2.1-3.0 cm; 4.3%, 8.6%, 8.6%, 10.9%, and 10.9% for tumor size 3.1-5 cm; and 0%, 0%, 0%, 12.0%, and 12.0% for tumor size >5 cm, respectively. PLT, platelet count.

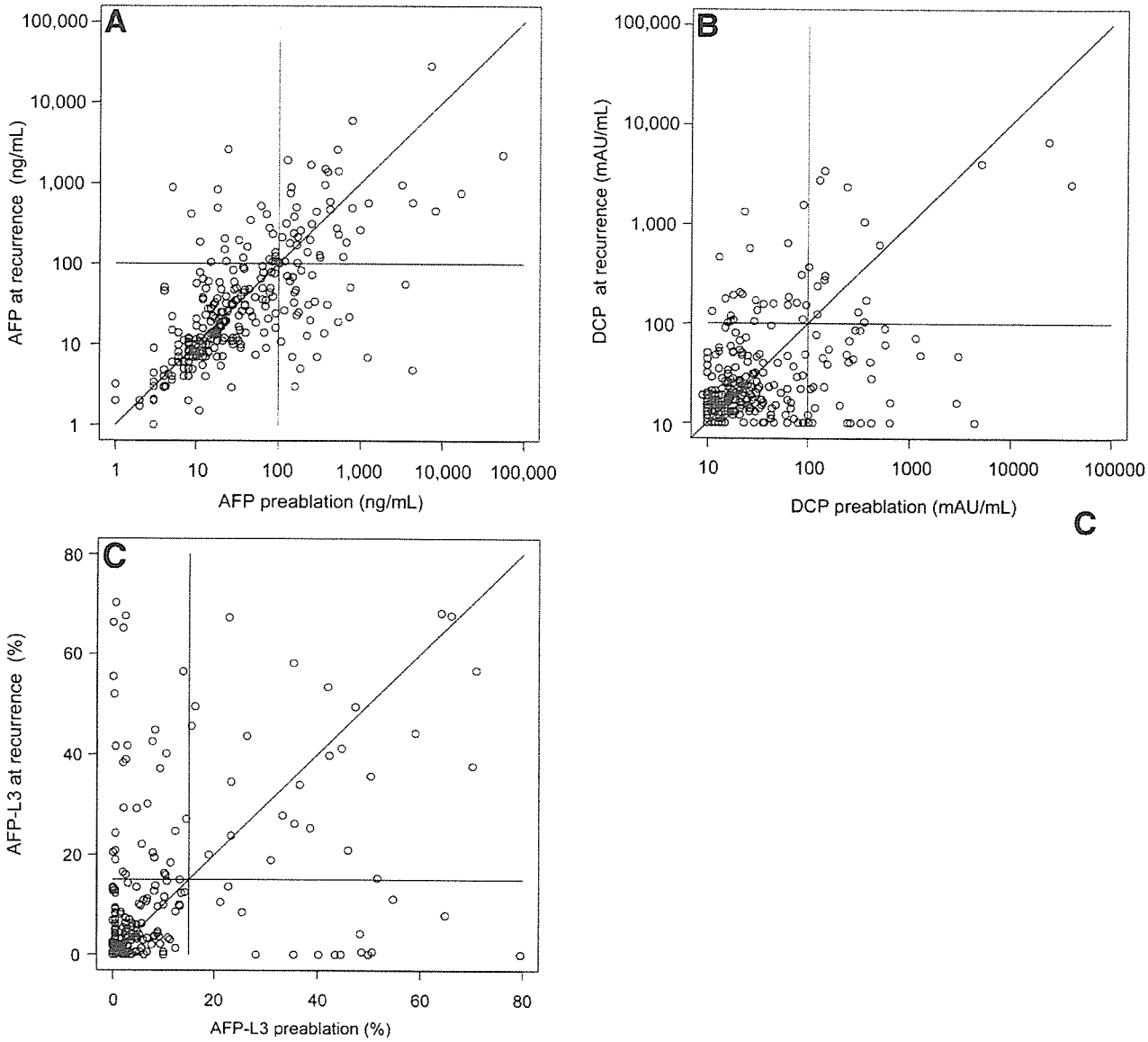

Fig. 4. Scatter plots of (A) alpha-fetoprotein, (B) des-gamma-carboxy prothrombin, and (C) lens culinaris agglutinin-reactive fraction of AFP preablation and at recurrence. AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

Discussion

In this study, a recurrence of HCC was extremely frequent, although each initial treatment had been judged complete. In fact, various studies have shown a similar or higher incidence of HCC recurrence after successful medical and surgical treatments.[34,35] It has been assumed that there are two distinct types of intrahepatic recurrence of HCC: *de novo* carcinogenesis and intrahepatic metastasis.[36] Those factors responsible for HCC development, such as fibrosis stage, age, sex, and presence of viral hepatitis, may also affect *de novo* carcinogenesis.[34,35] On the other hand, factors related to the primary tumor, such as the size and number of tumors, pathological grade, the presence of vascular invasion, and positivity of tumor markers, may affect the possibility of intrahepatic occult metastasis at the time of initial treatment.

We confirmed that AFP was independent from tumor size and number as a risk factor for recurrence. More than a few authors reported AFP as an independent predictor of poor prognosis, even in patients who had received curative resection.[21-23,37] High AFP values may indicate intra- or extrahepatic cancer spread and be associated with poor prognosis. On the other hand, it is well known that AFP may increase in some patients with acute or chronic

hepatitis without HCC.[38,39] It is also reported that AFP levels correlate with inflammation of background disease.[40] A high value of AFP postablation may also indicate active hepatic necrosis and subsequent regeneration, which may be associated with a high potential of *de novo* carcinogenesis in the background liver. The high level of AFP postablation is associated with a high risk of recurrence in at least two mechanisms, which are not easily distinguishable.

In the multivariate analysis on predictability of recurrence by tumor markers, we adopted a cutoff value of 100 ng/mL for AFP based on the distribution of AFP values pre- and postablation (Fig. 2A), where a large proportion of patients showed no changes in AFP levels through complete ablation when the preablation value was below 100 ng/mL. This may indicate that AFP was secreted mainly from the background liver rather than tumor itself when the level was below 100 ng/mL.[41,42]

This study revealed that AFP-L3 positivity after ablation was the strongest predictor of recurrence. Those whose AFP-L3 was more than 15% postablation all encountered recurrence within 1.5 years, except for one who died of liver failure without recurrence 1.1 years after ablation. AFP-L3 is a fucosylated variant of AFP that reacts with *Lens culinaris* agglutinin A, and elevation in the AFP level accompanied by an increase in AFP-L3 fraction is highly specific to HCC.[26,27,43] AFP-L3 positivity postablation strongly indicates residual cancer that cannot be detected with imaging techniques. More frequent checkups with dynamic CT may be beneficial for these patients. For overall prognosis, it is essential to detect recurrent HCC as early as possible via careful checkup and treat it effectively whenever possible, especially in countries such as Japan, where the application of liver transplantation is quite limited.

AFP-L3 value pretreatment has also been reported to be correlated with poorly differentiated cancer and poor prognosis.[44,45] In this study, AFP-L3 preablation was a significant predictor of recurrence in the multivariate analysis, but retained no significance in those whose AFP-L3 had decreased below 15% after ablation. This may indicate that ablation therapy is highly effective even for poorly differentiated HCC, and the poor prognosis of AFP-L3–positive HCC may be reversible if the marker can be turned negative by the complete ablation of tumor.

DCP, also known as prothrombin induced by vitamin K absence-II, is an abnormal prothrombin protein that is increased in the serum of HCC patients. Since the report by Liebman et al.,[24] DCP has been recognized not only as a highly specific marker for HCC, but also as a predictor of the prognosis of HCC patients.[46,47] In this study, DCP >100 mAU/mL preablation showed marginal significance in multivariate analysis for the predictability of recurrence, though it is significant in univariate analysis. In contrast to AFP and AFP-L3, DCP >100 mAU/mL preablation was significant in patients whose DCP value decreased below 100 mAU/mL after curative ablation. DCP did not turn negative in 10% of patients whose DCP value was >100 mAU/mL before ablation. In those patients, DCP positivity postablation may indicate something other than residual cancer. It is known that patients with severely impaired liver function show a high level of DCP even without HCC.[48] Indeed, patients with positive DCP postablation showed a high incidence of death without recurrence.

As diagnostic markers for HCC, the sensitivity of each of the three tumor markers was rather low in the present study because the patients had less-advanced disease suitable for curative ablation. However, the sensitivity of AFP-L3 had increased to 23.3% at the diagnosis of recurrence, from 13.7% before ablation, whereas sensitivity did not increase at recurrence in the other two markers. Fifteen percent of patients negative for AFP-L3 preablation turned positive at the time of recurrence. This may suggest that the diagnostic value of tumor markers increases during the clinical course of HCC.[45]

Recurrent tumor was sometimes detected adjacent to the primary site (local tumor progression), even if the treatment had been judged as complete on CT examination. We analyzed the risk of local tumor progression separately. The results showed that AFP >100 ng/mL pre- and postablation and AFP-L3 >15% postablation were significant risk factors for local tumor progression as well as for remote recurrence (data not shown). Local tumor progression and the majority of remote recurrence are similar in that they involve residual tumors undetectable via imaging evaluation of the treatment.

In conclusion, treatment efficacy in solid tumors has generally been based on findings obtained with imaging modalities.[49] Because AFP-L3 value after curative ablation assessed via imaging could predict residual cancer, tumor markers may be a complement end point for the treatment of HCC.

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LIVER TRANSPLANTATION 12:1077-1083, 2006

ORIGINAL ARTICLE

Selection of Liver-Transplant Candidates for Adult-to-Adult Living Donor Liver Transplantation as the Only Surgical Option for End-Stage Liver Disease

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The selection of living donor liver transplantation (LDLT) recipients in regions where deceased donor liver transplantation (DDLT) is rarely performed might be different from that in other centers at which LDLT is an alternative option to DDLT. Records of adult (age ≥ 18 yr) patients referred to our center were reviewed to analyze the selection process of LDLT candidates. Among the 533 LDLT candidates, 165 (31%) were rejected due to recipient issues. Advanced hepatocellular carcinoma (HCC) was the most common reason for rejection (n = 55). Among the remaining recipients, 120 patients (22%) were rejected due to donor issues. LDLT was eventually performed in 249 (47%) of the evaluated recipients. There are few options for candidates who are unable to find live donors in regions where DDLT is unrealistic. A more effective and precise approach to recipient and donor evaluation should be pursued. *Liver Transpl 12:1077-1083, 2006.* © 2006 AASLD.

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Advances in surgical techniques have led to a worldwide increase in living donor liver transplantation (LDLT).[1-6] Graft survival after LDLT among adult patients is comparable to that after deceased donor liver transplantation (DDLT).[7-9] The major advantages of performing LDLT over DDLT are a shorter recipient waiting time and therefore a lower rate of mortality while on the waiting list, a nonemergent surgical schedule, and a lower rate of ischemic injury to the donor organ. Donor safety is a concern in LDLT, with a mortality rate of 0.1 to 0.8%[10] and an early surgical complication rate of 12 to 29%.[11,12]

In Asia, deceased donor organs are extremely rare due to religious and cultural traditions. Thus, LDLT is increasingly performed, and in some Asian countries LDLT is performed more often than DDLT.[6,13] LDLT has led to a reduced mortality rate and a prolonged survival of selected adult patients with end-stage liver disease. In Japan, the first successful adult-to-adult LDLT was performed in 1993, and more than 300 adult-to-adult LDLTs were performed in 2003.[9] In contrast, only 28 DDLTs were performed from the time the Organ Transplant Law became effective in 1997 through August 2005.[14] Therefore, if the evaluation of a living donor is negative, few options remain for transplant candidates.

For conditions in which DDLT is unrealistic, patients

Abbreviations: LDLT, living donor liver transplantation; DDLT, deceased donor liver transplantation; HCC, hepatocellular carcinoma.

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TABLE 1. Patient Characteristics

Factors	Total (n = 533)	LDLT (n = 249)	Rejected (n = 284)	*P* value
Age (yr) (median, range)	51 (18-75)	51 (18-65)	51 (18-75)	0.258
Gender (men:women)	321:212	137:112	184:100	0.026
Indication				
Hepatocellular carcinoma	168	71	97	0.096
Primary biliary cirrhosis	66	51	15	<0.001
HCV cirrhosis	66	24	42	0.47
Fulminant hepatic failure	60	26	34	0.338
HBV cirrhosis	30	14	16	0.574
Biliary atresia	22	14	8	0.080
Autoimmune hepatitis	20	10	10	0.470
Primary sclerosing cholangitis	18	8	10	0.519
Cryptogenic cirrhosis	18	7	11	0.333
Hemophilia	14	6	8	0.494
Alcohol	13	1	12	0.003
Wilson's disease	5	3	2	0.438
HBV and HCV coinfection	2	0	2	0.283
Others	31	14	17	0.504

Abbreviations: LDLT, living donor liver transplantation; HCV, hepatitis C virus; HBV, hepatitis B virus.

and families place their last hope on the LDLT procedure, and come to the transplant program with high motivation. Public awareness continues to increase, and an increasing number of patients and potential donors are being seen at each transplant program. In an early report[15] regarding the patient and donor selection process that focused mainly on pediatric LDLT, 85% of evaluated patients underwent LDLT. Recent reports on adult recipients[16-23] are mainly from countries where the number of DDLTs is high. Indication criteria of LDLT both in donors and recipients might differ depending on the availability of deceased donor organs. In this article, we report our experience of evaluating patients and living donors for adult liver transplantation in a setting with scarce availability of deceased donor organs.

PATIENTS AND METHODS

Selection of Subjects

Between January 1996 and March 2005, 533 potential LDLT recipients were evaluated at the University of Tokyo adult-to-adult liver transplantation program. The median age was 51 (18-75) yr. Of the 533 potential recipients, 321 were men and 212 were women. The medical charts of all the candidates referred to our program were reviewed.

Selection of Recipients

Patients less than 65 yr of age and referred with a history of end-stage liver disease, fulminant hepatic failure, hepatocellular carcinoma (HCC), or metabolic or congenital liver disease were considered for liver transplantation. Institutional inclusion criteria for patients with HCC were as follows: no more than 5 nodules, each sized ≤5 cm. Extrahepatic metastasis and vascular invasion were contraindications for transplantation. Patients with alcoholic liver disease were required a 6-month period of abstinence.

The other conditions that were absolute contraindications for LDLT were pulmonary hypertension, uncontrollable cardiac failure, uncontrollable respiratory failure, and uncontrollable infectious disease. LDLT with ABO-incompatible donors for adult patients was not performed at our hospital. Indication for all evaluated candidates is shown in Table 1.

Selection of Donors for LDLT

Donor evaluation was scheduled primarily by the surgeon according to our center's protocol (Table 2). At the initial visit, a detailed presurgical process of evaluation, donor risk, and follow-up schedule were explained by the transplant physician, and a booklet explaining the donation procedure was given to each potential donor and recipient. All potential donors were interviewed independently by the transplant coordinator (registered nurse). Our criteria for living donors (listed in Table 2) were as follows: healthy individual between 20 and 65 yr of age; no significant medical history or abdominal surgery; and no history of viral hepatitis. Donor candidates within 3 degrees of consanguinity or the spouse of the recipient were considered for the operation, and any deviation from this criteria was discussed on a case-by-case basis by both the transplant team and the institutional ethics board.

After confirming the voluntary decision to become a live donor, the second phase of the evaluation was performed. This process included blood tests, a chest X-ray, electrocardiogram, and pulmonary function tests. For donor candidate older than 40 yr of age, serum

TABLE 2. Evaluation of Donor

- Phase 1
  - Interviewed by transplant coordinator
    - Confirmed of voluntarism becoming living donor
    - Age ≥ 20 and ≤ 65 yr
    - Within 3 degrees of consanguinity or spouse
    - Long-term relationship with recipient
  - Screened by transplant surgeons
    - No significant medical history
    - No viral hepatitis
    - ABO-compatible or identical
    - Consultation for psychiatric evaluation (if necessary)
- Phase 2
  - Laboratory tests
    - Complete blood count, blood type, hepatitis serology, liver function tests, coagulation, erythrocyte segimentation rate, urinalysis, iron, serum ferritin, transferring, ceruloplasmin, α1-antitrypsin level, and blood oxygen saturat ion, cytomegalovirus antibody (immunoglobulin G), Epstein-Barr virus antibody (immunoglobulin G), antinuculear antibody, human immunodeficiency antibody, adult T cell leukemia antibody, Alpha-fetoprotein, CEA
  - FOBT (age > 40 yr)
  - Chest X-ray, electrocardiogram, pulmonary function tests
  - Enhanced CT scan of abdomen
- Phase 3
  - Liver biopsy (when body mass index >25 and/or bright liver by abdominal ultrasound)
  - Gastrointestinal endoscopy (age > 40 yr)
  - Colonoscopy (when FOBT positive and/or CEA positive)
  - Treadmill test (age > 40 yr)
  - Three dimensional CT*
  - HLA typing/cross match
  - Indocyanine green retention rate at 15 minutes
  - Anesthesiology consultation
  - Final informed consent

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; CEA, carcinoemblionic antigen; CT, computed tomography; HLA, human leukocyte antigen; FOBT, fecal occult blood test.
*Performed since 2004, replacing angiography.

tumor markers and fecal occult blood test were added. Abdominal computed tomography scan with intravenous contrast was performed to rule out anatomic or medical contraindications.

If the donor cleared this phase, then the third phase began. Liver biopsy was performed when a fatty liver was suspected. A treadmill test and endoscopy were performed in donors older than 40 yr of age. Colonoscopy was also performed when a fecal occult blood test and/or serum carcinoembryonic antigen were positive. When eligibility was confirmed, angiography was performed as part of the third phase. In January 2004, angiography was replaced with three-dimensional computed tomography. Because of this change, most donor evaluation processes are now performed on an outpatient basis.

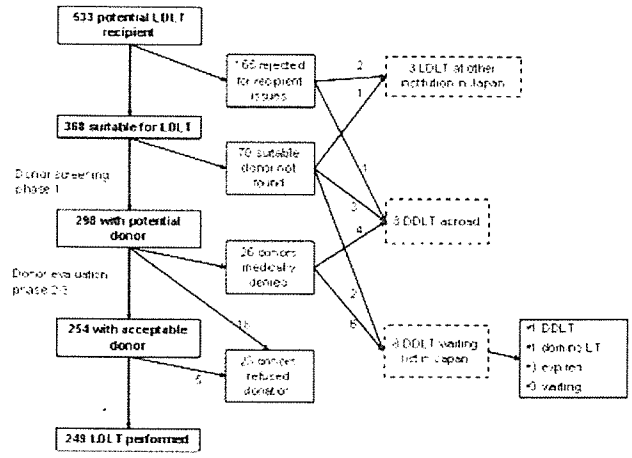

Figure 1. Among the 533 LDLT candidates, 165 (31%) were rejected due to recipient issues. Among the remaining recipients, 70 were unable to find suitable donor, 26 donors were medically denied, and 23 donors refused to donation. LDLT was eventually performed in 249 (47%) of the evaluated recipients. A small number of patients underwent DDLT or LDLT at other institute or abroad after being rejected at our center.

Ethical and Financial Consideration

At any time during the evaluation process potential donors could contact the transplant coordinator directly via telephone or e-mail, and express any conflicts or complaints. If necessary, potential donors were seen by unrelated surgeons, physicians, or psychiatrists for assessment of psychosocial problems.

The medical expenses of LDLT have been covered by national health insurance for patients with biliary atresia, Budd-Chiari syndrome, cholestatic liver diseases, Alagille syndrome, and congenital metabolic diseases. From 2004 onward, adult patients with fulminant hepatic failure, liver cirrhosis, hepatocellular carcinoma meeting the Milan criteria,[24] polycystic liver disease, and Caroli's disease were also added for insurance coverage. The expenses of donor evaluation and surgery were covered by insurance only if LDLT was performed for insurance-covered disorders. The costs of evaluation for rejected donors were not covered. All patient-donor pairs not meeting institutional and insurance criteria were reviewed by the institutional ethics board for final approval.

Statistical Analysis

Differences between groups were analyzed by independent-groups *t*-test for continuous variables and chi-squared test for categorical variables.

RESULTS

Patient Characteristics and Overall Outcome After Referral

A total of 533 potential LDLT recipients were evaluated (Table 1). Male patients were more frequently rejected than female patients ($P = 0.026$). Patients with primary

TABLE 3. Recipient Related Issues Unsuitable for LDLT

Reasons	N
Declined to undergo LDLT	15 (3)
Financial reasons	8 (2)
Medically not indicated	142 (48)
Advanced HCC	55 (15)
Advanced disease including multiorgan failure	28 (11)
Advanced age (>65 yr)	14 (5)
Too early for LDLT	12 (1)
Uncontrolled infectious diseases	11 (6)
Medical comorbidity unrelated to ESLD	10 (6)
Not abstaining from alcohol	1 (0)
Spontaneous recovery from FHF	8 (2)
Resectable HCC	1 (1)
Severe stenosis of hepatic artery	1 (1)
Noncompliance with treatment	1 (0)

NOTE: Numbers in parentheses indicate recipient candidates rejected after donor screening started.
Abbreviations: ESLD, end-stage liver disease; FHF, fulminant hepatic failure; LDLT, living donor liver transplantation; HCC, hepatocellular carcinoma.

TABLE 4. Donor Related Issues

Factors	N
Rejection at Phase 1 evaluation	70
No volunteer donor	45
Psychosocial problems	7
ABO incompatible	7
Marked physique discrepancy between recipient and donor	3
Under legal age	2
Over 65 yr old	2
Hepatitis B virus carrier	2
Significant medical history	2
Rejection at Phases 2 or 3 evaluation	26
Inappropriate liver volume	13
Steatosis >10%	7
Anatomical reason	1
Chronic hepatitis C, Colon cancer	1
Abnormal liver function	1
Respiratory disease	1
Pregnancy	1
Bronchial asthma, poorly controlled	1
Refusal to donate	23
Before or during medical evaluation	18
After evaluation	5

biliary cirrhosis had LDLT more frequently than others ($P < 0.001$). In contrast, patients with alcoholic liver disease were prone to be rejected ($P = 0.003$). Overall outcome of these patients is shown in Figure 1. Of these, 165 patients (31%) were rejected due to recipient issues. Among the 368 recipients who were deemed suitable for LDLT, 70 patients (19%) were unable to find suitable donors during the screening period. During further donor evaluation, 49 additional patients were excluded due to donor issues. As a result, 249 patients received LDLT at our hospital from their children (n = 106; 43%), siblings (n = 47; 19%), spouses (n = 44; 18%), parents (n = 33; 13%), or other individuals (n = 19; 7%).

Reasons for Rejection Due to Recipient Issues

The reasons for rejection due to recipient issues are summarized in Table 3. Fifteen patients declined to undergo LDLT, and 8 refused to undergo evaluation due to financial reasons.

A total of 142 patients were medically denied. Of those, 55 patients had HCC beyond our inclusion criteria; multiple nodules exceeding the institutional criteria without extrahepatic progression (n = 35), portal vein invasion (n = 8), extrahepatic metastasis (n = 7), rupture of HCC (n = 3), tumor seeding (n = 1), and invasion to inferior vena cava (n = 1).

A total of 28 patients were deemed too ill for LDLT. Usually, these patients had multiorgan failure other than liver disease at the time of referral. Of these, 17 died before the patient was transferred to our hospital due to the following reasons: varices rupture (n = 6), liver failure (n = 5), fulminant hepatic failure complicated with multiple organ failure (n = 4), intracranial bleeding (n = 1), and respiratory failure (n = 1). Ten patients with a volunteer donor died before completing donor evaluation due to liver failure. The remaining patient-donor pair was rejected due to pulmonary hypertension.

A total of 11 patients had uncontrolled infectious disorders: sepsis (n = 3), bacterial pneumonia (n = 2), fungal infection (n = 2), endocarditis (n = 1), sinusitis (n = 1), tuberculosis (n = 1), and *Pneumocystis carinii* pneumonia (n = 1). Another 10 had medical comorbidity unrelated to end-stage liver disease, including neoplasms (n = 5), uncontrolled psychiatric disorders (n = 2), severe obesity with uncontrolled adrenal disease (n = 1), interstitial pneumonia (n = 1), and benign hepatic tumor (n = 1).

Among the 12 rejected patients with alcoholic liver disease, only 1 patient was rejected because of failure to abstain from alcohol. Four were rejected for previously-referenced reasons; declination, varices rupture, too early, and medical comorbidity. The other 7 were rejected for donor issues.

Of the 169 patients who were rejected due to recipient-related issues, the initial screening of volunteer donors was started in 53 cases. The donor evaluation was discontinued as soon as the recipient withdrew.

Reasons for Rejection Due to Donor Issues

Reasons for rejection due to donor issues are summarized in Table 4. A total of 119 candidates were rejected due to donor issues. During phase 1, 70 patients were rejected; 45 had no volunteer donor and 25 had candidates not suitable for donor operation. During phases 2 and 3, donor suitability was considered medically con-