RESULTS

Mean follow-up was 7.5±3.4 years. During the follow-up period, 27 de novo malignancies were diagnosed in 26 liver transplant recipients (Table 1). Colorectal cancer was the most commonly detected malignancy (n=8) followed by gastric cancer and carcinoid (n=3 and 1, respectively), posttransplantation lymphoproliferative disorder (PTLD; n=3), leukemia (Langerhans cell sarcoma was included; n=3), skin cancer (Bowen's disease was included; n=2), oral and esophageal cancer (n=2), prostate cancer (n=2), renal cell cancer (n=2), and breast cancer (n=1). Among these, 7 of 27 (26%) recipients died from the de novo malignancy (Table 1). All but one gastrointestinal tract malignancy was diagnosed by screening endoscopy: esophageal cancer (1 of 1 [100%]), gastric cancer (one carcinoid; 4 of 4 [100%]), and colorectal cancer (7 of 8 [88%]). Seven of 13 (54%) were diagnosed with stage I (according to the tumor-node-metastasis classification) stomach or colorectal cancer. Among these, 5 of 7 (71%) were treated with endoscopic submucosal dissection. In total, 18 of 27 (59%) of de novo cancers were diagnosed as limited local, and surgical procedure including endoscopic submucosal dissection was applied to treat these cancers (Table 1).

Mean age of recipients diagnosed with de novo malignancy was 56 years. When expressed in terms of incidence per 100 person-years by age groups at the time of adult-to-adult LDLT, the rates were 0.6, 1.1, 0.2, 1.0, and 4.2 in the age groups of 18 to 29, 30 to 39, 40 to 49, 50 to 59, and 60 to 67 years, respectively (Fig. 1).

The subject of the study had similar sex and age distribution ratio with those of the Japanese population-based study. In our study, 59% (16 male, 11 female) was male and 89% (n=3 for the 20–39 years old and n=24 for the 40–74 years old) of de novo malignancy recipients were 40 and more than 40 years old at end of this study. Among the malignancy patients in a Japanese population-based study,

TABLE 1. Clinical characteristics of the 27 patients with de novo malignancies

	Age, sex	Primary disease	Diagnosis	Duration to diagnosis (yr)	Age at de novo malignancy	Treatment	Prognosis (death=1)
1	51/M	PBC	Oral	4.5	56	Radio	1
2	63/M	FHF	Esophageal	2.4	65	Chemo	1
3	64/M	PBC	Gastric	11.7	76	Resection[a]	0
4	51/M	PBC	Gastric	3.9	55	Resection[a]	0
5	52/M	LC (HBV), HCC	Gastric	1.8	54	ESD[a]	0
6	63/F	FHF	Gastric (carcinoid)	7.7	71	ESD[a]	0
7	64/F	FHF	Colorectal (cecum)	8.9	73	ESD[a]	0
8	62/F	PBC	Colorectal (ascending colon)	8.3	70	Resection[a]	0
9	54/F	PBC	Colorectal (ascending colon)	7.2	61	Resection[a]	0
10	60/M	LC (HBV)	Colorectal (ascending colon)	2.0	62	Chemo	1
11	57/F	AIH	Colorectal (sigmoid)	10.5	67	Resection[a]	0
12	55/F	LC (HBV), HCC	Colorectal (sigmoid)	4.5	60	Resection[a]	0
13	61/F	LC (HCV), HCC	Colorectal (rectal)	7.4	68	ESD[a]	0
14	56/M	LC (HCV), HCC	Colorectal (rectal)	5.6	62	ESD[a]	0
15	37/F	PBC, HCV	Breast	3.4	40	Resection[a]	0
16	63/M	LC (HCV), HCC	Prostate	5.4	68	Resection[a]	0
17	57/M	LC (HCV), HCC	Prostate	3.9	61	Resection[a]	0
18	39/F	PBC	RCC	4.1	43	Resection[a]	0
19	23/F	BA	RCC	1.6	24	Resection[a]	1
20	53/F	PBC	Skin (SCC)	14.7	68	Resection[a]	0
21	53/M	LC (HCV), HCC	Skin (Bowen's disease)	7.4	60	Resection[a]	0
22	25/M	PSC	PTLD	8.1	33	Resection+ chemo	0
23	62/M	LC (HCV), HCC	PTLD	6.0	68	Chemo	0
24	56/M	LC (HCV), HCC	PTLD	3.3	59	Chemo	1
25	49/F	PBC, HCC	Leukemia (Langerhans cell sarcoma)	4.4	53	Chemo	1
26	30/M	LC (HCV+HIV)	Leukemia	3.2	33	Chemo	0
27	60/M	FHF (drug)	Leukemia (acute myelogenous)	1.4	61	Chemo	1

[a] De novo malignancy was diagnosed as limited local.

AIH, autoimmune hepatitis; BA, biliary atresia; Chemo, chemotherapy; ESD, endoscopic submucosal dissection; FHF, fulminant hepatic failure; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; LC, liver cirrhosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; PTLD, posttransplantation lymphoproliferative disorder; RCC, renal cell carcinoma; Radio, radiotherapy; SCC, small cell carcinoma.

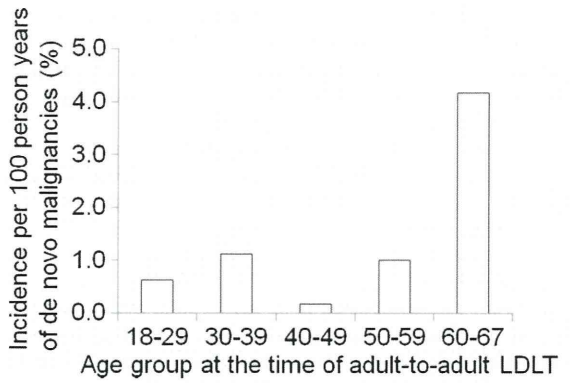

FIGURE 1. Incidence per 100 person-years by age groups at the time of adult-to-adult LDLT. Malignancies occurred most frequently in those 60 to 67 years old at liver transplantation (4.2/100 person-years) followed by those 30 to 39 years old (1.1) and then those 50 to 59 years old (1.0). LDLT, living-donor liver transplantation.

58% (253,210 male, 183,587 female) was male and 95% (n=22,312 for the 20–39 years old and n=414,485 for the 40–74 years old) of malignancy patients was 40 and more than 40 years old.

Overall mortality of transplant recipients with de novo malignancies was similar to findings of the Japanese general population-based study (standardized mortality ratio [SMR] = 0.9; 95% confidence incidence [CI], 0.4–2.0). Overall, the incidence of malignancy was significantly higher in transplant recipients than in the Japanese general population (SIR=1.8; 95% CI, 1.3–2.7). The risk of malignancy was slightly higher in female transplant recipients (SIR=1.9; 95% CI, 1.0–3.4) than in male recipients (SIR=1.8; 95% CI, 1.1–2.9; Table 2). The risk of malignancy was significantly higher in younger recipients than in the Japanese general population: 20 to 29 years old (SIR=48.0; 95% CI, 6.9–335.1), 30 to 39 years old (SIR=8.6; 95% CI, 2.2–34.1), and 40 to 49 years old (SIR=2.5; 95% CI, 0.6–9.9). The risk of malignancy was similar in older recipients: 50 to 59 years old (SIR=1.1; 95% CI, 0.4–3.0), 60 to 69 years old (SIR=1.1; 95% CI, 0.6–1.9), and 70 to 74 years (SIR=1.0; 95% CI, 0.4–2.5; Table 3). Malignancy sites or types with a significantly elevated SIR were as follows: head and neck (SIR=3.7; 95% CI, 0.5–26.6), esophagus (SIR=16.9; 95% CI, 2.4–17.9), stomach (SIR=1.6; 95% CI, 0.6–4.3), colorectal (SIR=3.5; 95% CI, 1.8–7.0) (*8*), prostate (SIR=2.2; 95% CI, 0.6–8.9), kidney (SIR=6.4; 95% CI, 1.6–25.4), malignant lymphoma (SIR=7.6; 95% CI, 2.5–23.6) (*9*), and leukemia (SIR=15.1; 95% CI, 4.9–46.9) but not breast (SIR=0.9; 95% CI, 0.1–6.4; Table 4).

The 3-, 5-, and 10-year estimated survival rates of recipients with de novo malignancies were 93%, 81%, and 57%, respectively, and those in recipients without de novo malignancies were 95%, 93%, and 92%, respectively (P=0.0001). The cumulative incidence of de novo malignancies at 3, 5, and 10 years after transplantation was 2%, 5%, and 10%, respectively. After de novo malignancies were diagnosed, the 1-, 3-, and 5-year estimated survival rates were 81%, 69%, and 61%, respectively.

TABLE 2. Total mortality rates and SMRs with 95% CI and total, male, and female IRs (×100,000) and SIRs with 95% CI

	n	IR (×100,000)	SIR	95% CI
Total mortality	7	259	0.9	0.4–2.0
Total incidences	27	963	1.8	1.3–2.7
Male	16	1085	1.8	1.1–2.9
Female	11	850	1.9	1.0–3.4

CI, confidence interval; IR, incidence rate; SIR, standardized incidence ratio; SMR, standardized mortality ratio.

TABLE 3. IRs (×100,000) and SIRs with 95% CI according to age

Age, yr	n	IR (×100,000)	SIR	95% CI
20–29	1	169	48.0	6.9–335.1
30–39	2	78	8.6	2.2–34.1
40–49	2	60	2.5	0.6–9.9
50–59	4	45	1.1	0.4–3.0
60–69	14	115	1.1	0.6–1.9
70–74	4	172	1.0	0.4–2.5

CI, confidence interval; IR, incidence rate; SIR, standardized incidence ratio.

DISCUSSION

Continuous improvements in surgical techniques and immunosuppression regimens have greatly improved the long-term results of LDLT. We reported the cause of death in 176 adult-to-adult LDLT recipients in 2005 with a median follow-up period of 2.8 years and concluded that recurrent primary disease, infection, and surgical complications in bile duct anastomosis impact the long-term outcome (*10*). Similar to the long-term findings of deceased-donor liver transplantation recipients, however, de novo malignancies were the main cause of death. According to previous reports (*11–15*), the overall risk of malignancy is two to four times higher in transplant recipients than in an age- and sex-matched population. In our

TABLE 4. IRs (×100,000) and SIRs with 95% CI according to site or type of malignancy

Malignancy	n	IR (×100,000)	SIR	95% CI
Head and neck	1	4	3.7	0.5–26.6
Esophagus	1	4	16.9	2.4–17.9
Stomach	4	15	1.6	0.6–4.3
Colorectal	8	30	3.5	1.8–7.0
Breast	1	8	0.9	0.1–6.4
Prostate	2	14	2.2	0.6–8.9
Kidney	2	7	6.4	1.6–25.4
Skin	2	7	6.4	1.6–25.4
Malignant lymphoma	3	11	7.6	2.5–23.6
Leukemia	3	11	15.1	4.9–46.9

CI, confidence interval; IR, incidence rate; SIR, standardized incidence ratio.

cohort, the risk of malignancy was similar to that in previous reports. Compared with the results of the general Japanese population-based study, however, the standardized mortality ratio (SMR) was 0.9.

Regarding the evaluation of the extent of the malignancy at diagnosis, in our cohort, 18 of 27 (67%) of de novo malignancies were diagnosed as limited local. According to the general Japanese population-based study, although there were no detailed stage data by the tumor-node-metastasis classification, 35% of malignancy was limited local, 21% was invaded to adjacent organ or lymph node metastasis, and 14% had distant metastasis at diagnosis (the remaining 30% was unknown) (*7*). One of the reasons for early malignancy diagnosis in our cohort might be malignancy screening rate. The rate of malignancy screening of Japanese general population was 21% to 26%, which was disclosed in public by Ministry of Health, Labour and Welfare of Japan (*16*). The present study was biased by the small number of patients; however, a malignancy surveillance protocol might reduce mortality in this cohort.

Younger recipients had high risk for de novo cancer in our study. There was a difference of type of malignancy between the Japanese population-based study and our cohort. In the Japanese population-based study, in the younger population (20–39 years old), the most frequent malignancy was uterus, and the second to fifth most frequent were breast, stomach, colorectal, and thyroid cancer, respectively, which accounts for 70% of the younger malignancy population (*7*). In our study, three younger (20–39 years old) recipients developed malignancy. The type of malignancy of these recipients consisted of breast cancer, PTLD, and renal cell cancer. It is well known that younger recipients have risk (*17*) of PTLD in solid organ transplant recipients. However, further study is needed because of the small number of younger recipients in our study.

Malignancy types differ between races. In a western study, Buell and colleagues reported that nonmelanocytic skin cancers are the most commonly reported de novo malignancy in solid organ transplant recipients, with the incidence varying in proportion to the degree of sun exposure (*18–20*). In Asian countries, including our study, skin cancer is less frequent. There are only a few reports from Asian countries. In a Korean liver transplant recipient study, stomach cancer was most frequent with a relative risk more than 10-fold higher than that in the general Korean population (*21*). In a Japanese population of renal transplant recipients, the most frequent malignancy was stomach and colorectal cancer when native renal cell cancer was excluded (*22*). In our cohort, the most frequent malignancy was colorectal cancer. The next most common malignancies were stomach cancer and malignant lymphoma. Two of the recipients in our cohort were diagnosed with skin cancer. Colorectal and stomach cancers might be the main malignancies in Asia. On the contrary, Penn reported that the average time to first malignancy was 5.0 years (*23*). In recent reports, Harwood reported skin cancer in organ transplant recipients with a 22-year prospective study. In their report, the median time to first skin cancer was 7.6 years (≥60 years old) to 24.1 years (30–39 years old) (*24*). In our study, mean follow-up time was 7.5 years. Our study may still underrepresent skin cancer risk.

Despite the high risk of de novo malignancy for recipients during the follow-up period, there is no consensus regarding the appropriate malignancy screening program after liver transplantation. Herrero and colleagues suggested that deceased-donor liver transplantation recipients should be screened periodically for malignancies common to the general population, which may result in timely detection of de novo malignancies (*25*). Our screening methods that focus on gastrointestinal and colorectal cancers might be suitable because prognosis after diagnosis with malignancy was relatively favorable (61% at 5 years). Our findings regarding the prognosis seem to be higher than that in previous reports. Herrero (*5*) reported that the 5-year prognosis after diagnosis of de novo malignancy in 51 liver transplant recipients diagnosed with a noncutaneous malignancy was approximately 40%. Age at diagnosis of malignancy is inversely related to the ratio of PTLD as a de novo malignancy (*26*). In our cohort, none of the younger recipients (<55 years old) who were diagnosed with malignancy had colorectal or stomach cancer. The three youngest recipients were diagnosed with renal cell cancer (24 years old), PTLD (33 years old), and Burkitt's leukemia (33 years old). Our screening methods might thus not be suitable for younger recipients.

The incidence of colon cancer in liver transplant recipients was initially thought to be similar to that in the general population (*27, 28*). A meta-analysis study, however, reported a relative risk of 2.6 for colorectal cancer in post–deceased-donor liver transplantation patients compared with an age-matched general population (*29*). Primary sclerosing cholangitis is an important high-risk factor for colorectal cancer. For example, Vera and colleagues (*30*) found a 5% incidence of colorectal cancers in recipients with primary sclerosing cholangitis versus 0.6% for recipients without nonprimary sclerosing cholangitis. Nicolaas and colleagues reported that overall transplant recipients (nonprimary sclerosing cholangitis) have an increased risk for colorectal cancer compared with the general population (relative risk: 1.8) (*29*). Thereby, they concluded that nonprimary sclerosing cholangitis transplant recipients do not need an intensified screening strategy for colorectal cancer. Based on our findings of a relatively high rate of colorectal cancer and of malignancy in recipients more than 60 years of age, we think that an active malignancy surveillance program for colorectal cancer might be needed for liver transplant recipients, especially those more than 60 years old and Asian. In the present study, 88% of colorectal cancers were diagnosed by screening colonoscopy. This study is a single-institution experience and a relatively small cohort. Further studies with a larger cohort of Japanese and/or Asian recipients are needed.

Colorectal malignancies predominated in Japanese liver transplant recipients. Although de novo malignancies correlated with a poor prognosis, the SMR was 0.9 compared with the Japanese population-based study.

MATERIALS AND METHODS

Between January 1996 and July 2012, 412 adult-to-adult LDLTs were performed at the University of Tokyo Hospital. The subjects of the present study were 360 adult LDLT recipients who survived more than 1 year after transplantation and had no previous diagnosis of malignancy, excluding hepatocellular carcinoma, at the time of transplantation. The indications

for transplantation-included hepatitis B or C–related cirrhosis (n=161), cholestatic liver disease (n=98), fulminant hepatic failure (n=38), biliary atresia (n=19), alcoholic liver cirrhosis (n=11), metabolic diseases (n=10), and others (n=23). Mean model for end-stage liver disease score (*31*) was 14.8±7.6. Mean recipient age was 49 years when transplantation was performed. The number of male and female recipients was 192 and 168, respectively. The mean age was 56 years when this study was performed. The age distribution was as follows: 20 to 29 years (3%; n=10), 30 to 39 years (8%; n=30), 40 to 49 years (13%; n=47), 50 to 59 years (28%; n=99), 60 to 69 years (41%; n=149), and 70 to 79 years (7%; n=25).

Screening examinations were performed as a first step in evaluating recipient candidates to exclude malignancy. Abdominal computed tomography or magnetic resonance imaging, upper gastrointestinal endoscopy, total colonoscopy, and several tumor markers (e.g., carcinoembryonic antigen, carbohydrate antigen 19-9, and prostate specific antigen tests) were examined. When malignancy other than hepatocellular carcinoma was found, preparation for transplantation was discontinued and treatment was started.

The transplantation procedure and donor selection criteria are described elsewhere (*32, 33*). All survivors were followed in our outpatient clinic through the end of July 2012. Mean follow-up period was 7.5±3.4 years.

The study protocol was approved by the University of Tokyo Ethics Committee (No. 2317).

Immunosuppression

Basic immunosuppressive agents, tacrolimus and methylprednisolone, were used. The target trough serum level of tacrolimus was 15 to 20 ng/mL in week 1 after transplantation. Simultaneously, methylprednisolone (20 mg/kg) was used before the anhepatic phase of surgery. Six months after surgery, the target tacrolimus trough level was gradually decreased from 8 to 5 ng/mL. At the same time, the dose of methylprednisolone was subsequently reduced to the maintenance level (0.05 mg/kg) (*34*). In patients who developed reversible posterior leukoencephalopathy syndrome as a side effect (*35*), tacrolimus was replaced with cyclosporine therapy. Acute and chronic rejection was diagnosed using the Banff schema classification (*36, 37*). When acute rejection was diagnosed, patients were treated with a bolus of intravenous methylprednisolone.

Malignancy Surveillance Program After Liver Transplantation

For patient management in the outpatient clinic, we recommend that patients undergo an annual medical checkup provided by their company or municipal government in accordance with the ordinance of the Ministry of Health, Labor, and Welfare of Japan. These medical checkups include a chest X-ray, gastrointestinal X-ray examination, stool occult blood for patients more than 40 years old, and/or breast physical examination (palpitation and mammography), and uterine cervical smears in women more than 40 years old. Additionally, we performed screening examinations as follows: abdominal computed tomography or magnetic resonance imaging, upper gastrointestinal endoscopy, and total colonoscopy and immunochemical fecal occult blood test every 1 to 2 years. Complete blood count and liver function tests with tumor markers (carcinoembryonic antigen, carbohydrate antigen 19-9, and prostate specific antigen tests) were performed every 1 to 3 months after LDLT. The diagnosis of de novo malignancy was based on histologic examination of obtained biopsies or surgical specimens of the tumors. The date of malignancy diagnosis was defined as the date of initial pathologic confirmation.

Statistical Analysis

As for incidence per 100 person-years of de novo malignancies by age group at the time of adult-to-adult LDLT, person-year was calculated at the end of July 2012 (total of 2705 person-years). When de novo malignancy was developed, patient was classified based on the age at the time of liver transplantation.

The estimated malignancy incidence and incidence rate in the Japanese general population was adopted from published data (*7*). The ratio of observed to expected number of malignancies, the SMR, and the SIR were calculated by dividing the observed number of LDLT recipients with malignancies by the expected number of malignancy patients (actual number of recipients multiplied by mean follow-up period divided by malignancy mortality (or incidence) rate of the 2006 Japanese population-based study (*7*). The 95% CI of SMR and SIR were determined using the Poisson distribution with Excel 2010 software (Microsoft Japan, Tokyo, Japan).

For comparison with a Japanese population-based malignancy study, we obtained published data available on a Web site (http://ganjoho.jp/professional/statistics/index.html) (*7*). Kaplan–Meier life table analysis with a log-rank test was used to assess whether de novo malignancies significantly affected posttransplantation patient survival using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Data are expressed as mean±standard deviation. $P<0.05$ was considered to be statistically significant.

REFERENCES

1. Hashikura Y, Makuuchi M, Kawasaki S, et al. Successful living-related partial liver transplantation to an adult patient. *Lancet* 1994; 343: 1233.
2. Penn I. Cancers in renal transplant recipients. *Adv Ren Replace Ther* 2000; 7: 147.
3. Vogt DP, Henderson JM, Carey WD, et al. The long-term survival and causes of death in patients who survive at least 1 year after liver transplantation. *Surgery* 2002; 132: 775.
4. Collett D, Mumford L, Banner NR, et al. Comparison of the incidence of malignancy in recipients of different types of organ: a UK Registry audit. *Am J Transplant* 2010; 10: 1889.
5. Herrero JI. De novo malignancies following liver transplantation: impact and recommendations. *Liver Transpl* 2009; 15: S90.
6. Cormier JN, Xing Y, Ding M, et al. Ethnic differences among patients with cutaneous melanoma. *Arch Intern Med* 2006; 166: 1907.
7. Matsuda T, Marugame T, Kamo K, et al. Cancer incidence and incidence rates in Japan in 2006: based on data from 15 population-based cancer registries in the monitoring of cancer incidence in Japan (MCIJ) project. *Jpn J Clin Oncol* 2012; 42: 139.
8. Malinchoc M, Kamath PS, Gordon FD, et al. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. *Hepatology* 2000; 31: 864.
9. Sugawara Y, Makuuchi M, Takayama T, et al. Safe donor hepatectomy for living related liver transplantation. *Liver Transpl* 2002;8:58.
10. Sugawara Y, Makuuchi M, Sano K, et al. Vein reconstruction in modified right liver graft for living donor liver transplantation. *Ann Surg* 2003; 237: 180.
11. Sugawara Y, Tamura S, Kaneko J, et al. Positive lymphocytotoxic crossmatch does not adversely affect survival in living donor liver transplantation. *Dig Surg* 2009; 26: 482.
12. Lanzino G, Cloft H, Hemstreet MK, et al. Reversible posterior leukoencephalopathy following organ transplantation. Description of two cases. *Clin Neurol Neurosurg* 1997; 99: 222.
13. Demetris A, Adams D, Bellamy C, et al. Update of the International Banff Schema for Liver Allograft Rejection: working recommendations for the histopathologic staging and reporting of chronic rejection. An international panel. *Hepatology* 2000; 31: 792.
14. Banff schema for grading liver allograft rejection: an international consensus document. Hepatology 1997; 25: 658.
15. Kazama S, Hongo K, Sunami E, et al. Six cases of primary colorectal cancer after living-donor liver transplantation: a single-institution experience in Japan. *Jpn J Clin Oncol* 2012; 42: 586.
16. Kataoka K, Seo S, Sugawara Y, et al. Post-transplant lymphoproliferative disorder after adult-to-adult living donor liver transplant: case series and review of literature. *Leuk Lymphoma* 2010; 51: 1494.
17. Hashimoto T, Sugawara Y, Kishi Y, et al. Long-term survival and causes of late graft loss after adult-to-adult living donor liver transplantation. *Transplant Proc* 2005; 37: 4383.
18. Herrero JI, Lorenzo M, Quiroga J, et al. De novo neoplasia after liver transplantation: an analysis of risk factors and influence on survival. *Liver Transpl* 2005; 11: 89.
19. Haagsma EB, Hagens VE, Schaapveld M, et al. Increased cancer risk after liver transplantation: a population-based study. *J Hepatol* 2001; 34: 84.
20. Sheiner PA, Magliocca JF, Bodian CA, et al. Long-term medical complications in patients surviving > or = 5 years after liver transplant. *Transplantation* 2000; 69: 781.

21. Oo YH, Gunson BK, Lancashire RJ, et al. Incidence of cancers following orthotopic liver transplantation in a single center: comparison with national cancer incidence rates for England and Wales. *Transplantation* 2005; 80: 759.
22. Aberg F, Pukkala E, Hockerstedt K, et al. Risk of malignant neoplasms after liver transplantation: a population-based study. *Liver Transpl* 2008; 14: 1428.
23. Ministry of Health, Labour and Welfare of Japan. Available at: http://www.mhlw.go.jp/shingi/2008/03/s0301-4.html. Accessed December 13, 2012.
24. Schober T, Framke T, Kreipe H, et al. Characteristics of early and late PTLD development in pediatric solid organ transplant recipients. *Transplantation* 2013; 95: 240.
25. Dreno B, Mansat E, Legoux B, et al. Skin cancers in transplant patients. *Nephrol Dial Transplant* 1998; 13: 1374.
26. Gupta AK, Cardella CJ, Haberman HF. Cutaneous malignant neoplasms in patients with renal transplants. *Arch Dermatol* 1986; 122: 1288.
27. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. *N Engl J Med* 2003; 348: 1681.
28. Park HW, Hwang S, Ahn CS, et al. De novo malignancies after liver transplantation: incidence comparison with the Korean cancer registry. *Transplant Proc* 2012; 44: 802.
29. Ishikawa N, Tanabe K, Tokumoto T, et al. Clinical study of malignancies after renal transplantation and impact of routine screening for early detection: a single-center experience. *Transplant Proc* 2000; 32: 1907.
30. Penn I. Cancers complicating organ transplantation. *N Engl J Med* 1990; 323: 1767.
31. Harwood CA, Mesher D, McGregor JM, et al. A surveillance model for skin cancer in organ transplant recipients: a 22-year prospective study in an ethnically diverse population. *Am J Transplant* 2013; 13: 119.
32. Herrero JI. Screening of de novo tumors after liver transplantation. *J Gastroenterol Hepatol* 2012; 27: 1011.
33. Fung JJ, Jain A, Kwak EJ, et al. De novo malignancies after liver transplantation: a major cause of late death. *Liver Transpl* 2001; 7: S109.
34. Vallejo GH, Romero CJ, de Vicente JC. Incidence and risk factors for cancer after liver transplantation. *Crit Rev Oncol Hematol* 2005; 56: 87.
35. Silva MA, Jambulingam PS, Mirza DF. Colorectal cancer after orthotopic liver transplantation. *Crit Rev Oncol Hematol* 2005; 56: 147.
36. Sint Nicolaas J, de Jonge V, Steyerberg EW, et al. Risk of colorectal carcinoma in post-liver transplant patients: a systematic review and meta-analysis. *Am J Transplant* 2010; 10: 868.
37. Vera A, Gunson BK, Ussatoff V, et al. Colorectal cancer in patients with inflammatory bowel disease after liver transplantation for primary sclerosing cholangitis. *Transplantation* 2003; 75: 1983.

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ORIGINAL ARTICLE

Significance of serum and hepatic microRNA-122 levels in patients with non-alcoholic fatty liver disease

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is believed to be a type of metabolic syndrome. MicroRNA-122 (miR-122) is the most abundant microRNA in the liver and is an important factor for the metabolism of glucose and lipids. In the present study, we examined the correlation between the hepatic and serum miR-122 expression levels and the clinicopathological factors of patients with NAFLD. *Methods:* We extracted the total RNA, along with preserved miRNAs, from liver biopsy samples of 67 patients with NAFLD. In 52 of these 67 patients, the total RNA was extracted from serum. The miR-122 that was obtained by quantitative reverse transcription-polymerase chain reaction was quantified using TaqMan MicroRNA assays. *Results:* A significant correlation was detected between serum and hepatic miR-122 expression (correlation coefficient, 0.461; $P = 0.005$). Patients with mild steatosis (<33%) showed significantly lower levels of hepatic miR-122 compared with patients with severe steatosis (>33%) (hepatic miR-122: mild/severe = 2.158 ± 1.786/4.836 ± 7.506, $P = 0.0473$; serum miR-122: mild/severe = 0.002 ± 0.005/0.007 ± 0.001, $P = 0.0491$). Moreover, hepatic and serum miR-122 levels were significantly higher in patients with mild fibrosis than in those with severe fibrosis (hepatic miR-122: mild/severe = 5.201 ± 7.275/2.394 ± 1.547, $P = 0.0087$; serum miR-122: mild/severe = 0.008 ± 0.011/0.002 ± 0.004, $P = 0.0191$). *Conclusions:* We found that the hepatic and serum miR-122 levels were associated with hepatic steatosis and fibrosis. The serum miR-122 level can be a useful predictive marker of liver fibrosis in patients with NAFLD.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide (1–8). NAFLD is considered to represent the hepatic manifestation of metabolic syndrome. In Japan, an increase in the incidence of metabolic syndrome has led to an increase in the prevalence of NAFLD (5). NAFLD was traditionally considered as a relatively benign liver disease. However, some patients with NAFLD progress to liver fibrosis, cirrhosis and hepatocellular carcinoma (8–13). Therefore, the precise diagnosis and staging of NAFLD patients is clinically important. Liver biopsy is the gold standard for the evaluation of NAFLD patients in terms of staging. However, liver biopsy is an invasive technique, and the identification of non-invasive biomarkers is required.

Micro-RNAs (miRNAs) are endogenous, small, non-coding RNAs of approximately 21–22 nucleotides that have important gene regulatory functions in animals and plants. miRNAs bind to the messenger RNAs of protein coding genes to direct their post-transcriptional repression (14–16). miRNAs have been reported to play important roles in cell proliferation (17) and apoptosis (18), lymphocyte development (19), and adipocyte differentiation (20). Several recent studies have indicated that miRNAs play important roles in metabolism and metabolic diseases (21–23). MicroRNA-122 (miR-122) is the most abundant miRNA in the liver, and it regulates metabolic pathways, including cholesterol biosynthesis, fatty acid synthesis and oxidation (22, 23).

Recently, extracellular miRNAs were detected in serum, plasma and other body fluids. These circulating miRNAs have been reported to be predictive biomarkers for various cancers and in liver diseases (24, 25). However, the significance of miR-122 expression in the serum and liver of NAFLD patients has not been studied in detail.

In the present study, we analysed the relationship between the clinicopathological features and the expression of miR-122 in the serum and liver of NAFLD patients.

Patients and methods

Patient groups

In this study, we examined consecutive NAFLD patients who visited the Department of Gastroenterology and Hepatology at Nagasaki University Hospital. The patients who exhibited positive results for hepatitis B virus surface antigen or hepatitis C virus antibody, or those showing evidence of inherited, autoimmune, cholestatic or drug-induced liver disease were excluded using clinical, laboratory, imaging and histological criteria. In addition, patients with a history of current or past excessive alcohol intake, as defined by an average daily consumption of more than 20 g of alcohol, were excluded from the study.

Non-alcoholic fatty liver disease was diagnosed by percutaneous liver biopsy and ultrasonography. Liver biopsy specimens were fixed in 10% formalin, cut to a thickness of 4 μm and subjected to haematoxylin–eosin and Azan-Mallory staining. Steatosis was classified as mild (>30%) or severe (30%). Inflammation was scored on a scale of 0–9 according to the standards proposed by the Non-alcoholic Steatohepatitis Clinical Research Network (26). Fibrosis staging was performed using a five-grade scale as follows: F0, no fibrosis; F1, pericellular fibrosis in zone 3; F2, pericellular fibrosis in zone 3 with periportal fibrosis; F3, bridging fibrosis; and F4, cirrhosis defined as mild fibrosis (F0 or F1) and severe fibrosis (>F1).

miRNA extraction and quantification

RNA was extracted from a total of 67 liver biopsy specimens. Total RNA, including the miRNA, was isolated from formalin-fixed paraffin-embedded (FFPE) liver biopsy specimens using the Recover All Total Nucleic Acid Isolation Kit for FFPE (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol. In 52 of 67 patients, total RNA, along with preserved miRNAs, was extracted from 400 μL of serum using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Synthetic miR-39 was added to serum samples prior to RNA extraction as an internal control.

The miR-122 obtained by quantitative reverse transcription-polymerase chain reaction was quantified using TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. miR-122 expression was calculated by the relative standard curve method and normalized to RNU6 expression in the liver and cell-miR39 expression in the serum.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analysed by the Student's *t*-test for comparison of paired data. Correlations were analysed using the Spearman rank correlation coefficient. A P value of <0.05 was considered statistically significant.

Results

The characteristic of this study population are show in Table 1.

Correlation between hepatic and serum miR-122 expression and clinical factors

No significant correlations were observed between clinical factors and the expression of hepatic (Table 2) or serum (Table 3) miR-122. However, a significant correlation was observed between the serum and hepatic miR-122 expression levels (Fig. 1).

Correlation between hepatic miR-122 level and the pathological findings of NAFLD patients

Patients with mild steatosis (<33%) showed significantly lower levels of hepatic miR-122 than patients with severe steatosis (>33%) (mild/severe = 2.158 ± 1.786/4.836 ± 7.506; $P = 0.0473$). No significant correlation between serum miR-122 level and the NAFLD activity score (NAS) was observed. In contrast, hepatic miR-122 level showed a significant negative correlation with the fibrosis stage [correlation coefficient: −0.292 (−0.497 to −0.056); $P = 0.0161$] (Table 2). Moreover, hepatic miR-122 expression was significantly higher in patients with no or mild fibrosis than in those with severe fibrosis (mild/severe = 5.201 ± 7.275/2.394 ± 1.547; $P = 0.0087$) (Fig. 2).

Correlation between serum miR-122 level and the pathological findings of NAFLD patients

Patients with mild steatosis (<33%) showed significantly lower levels of serum miR-122 than patients with severe steatosis (>33%) (mild/severe = 0.002 ± 0.005/0.007 ± 0.001; $P = 0.0491$). No significant correlation was

Table 1. Clinical characteristics of liver samples (67 cases)

Patient age (years)	51.8 ± 17.4
Male:female	27:40
BMI	28.5 ± 4.2
Type 2 diabetes	46 cases
AST (IU/L)	71.7 ± 42.4
ALT (IU/L)	102.7 ± 64.1
ALP (IU/L)	286.3 ± 117.3
γ-GTP (IU/L)	103.6 ± 121.6
T-cho (mg/dl)	195.1 ± 45.4
TG (mg/dl)	144.7 ± 60.1
Plt (10^4/mm^3)	21.7 ± 7.4
FBS (mg/dl)	115.7 ± 41.4
HbA1c (%)	6.7 ± 2.0

γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, free blood sugar; HbA1c, glyco haemoglobin A1c; Plt, platelet; T-cho, total cholesterol; TG, triglyceride.

Table 2. Relation between hepatic microRNA-122 level and clinical factors

	Correlation coefficient	*P*-value
Age	0.025 (−0.216 to 0.264)	0.8385
BMI	−0.107 (−0.342 to 0.141)	0.3984
AST	−0.142 (−0.369 to 0.102)	0.2541
ALT	−0.042 (−0.279 to 0.201)	0.7390
ALP	−0.072 (−0.307 to 0.142)	0.5657
γ-GTP	−0.082 (−0.318 to 0.163)	0.5125
T-cho	0.054 (−0.199 to 0.300)	0.6785
TG	0.125 (−0.119 to 0.354)	0.3152
Plt	0.123 (−0.121 to 0.352)	0.3422
FBS	0.224 (−0.034 to 0.454)	0.0878
HbA1c	0.250 (−0.017 to 0.483)	0.0660
NAS	0.053 (−0.190 to 0.289)	0.6732
Fibrosis	−0.292 (−0.497 to −0.056)	0.0161

γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, free blood sugar; HbA1c, glyco haemoglobin A1c; NAS, NAFLD activity score; Plt, platelet; T-cho, total cholesterol; TG, triglyceride.

Table 3. Relation between serum microRNA-122 level and clinical factors

	Correlation coefficient	*P*-value
Age	−0.183 (−0.434 to 0.095)	0.1959
BMI	−0.042 (−0.314 to 0.236)	0.7708
AST (IU/L)	−0.049 (−0.317 to 0.386)	0.7340
ALT (IU/L)	0.126 (−0.152 to 0.136)	0.3750
ALP (IU/L)	−0.143 (−0.400 to 0.136)	0.3146
γ-GTP (IU/L)	−0.125 (−0.387 to 0.156)	0.3849
T-cho	0.089 (−0.194 to 0.358)	0.5420
TG	−0.061 (−0.329 to 0.215)	0.6667
Plt	−0.035 (−0.305 to 0.240)	0.8044
FBS	0.212 (−0.087 to 0.476)	0.1626
HbA1c	0.114 (−0.193 to 0.401)	0.4695
NAS	0.138 (−0.140 to 0.396)	0.3312
Fibrosis	−0.316 (−0.543 to 0.048)	0.0218

γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, free blood sugar; HbA1c, glyco haemoglobin A1c; NAS, NAFLD activity score; Plt, platelet; T-cho, total cholesterol; TG, triglyceride.

detected between serum miR-122 levels and the NAS. Serum miR-122 expression in the liver showed a significant inverse correlation with fibrosis stage [correlation coefficient: −0.316 (−0.543 to 0.048); $P = 0.0218$] (Table 3). Moreover, serum miR-122 levels were significantly higher in patients with mild fibrosis than in those with severe fibrosis (mild/severe = 0.008 ± 0.011/0.002 ± 0.004; $P = 0.0191$) (Fig. 3).

To compare the ability of the blood tests to predict the fibrotic stage, we constructed receiver operating characteristics (ROC) curves for serum miR-122, hyaluronic acid and type IV collagen; the area under the ROC curves for miR-122, hyaluronic acid and type IV collagen were 0.82, 0.74 and 0.72, respectively (Fig. 4).

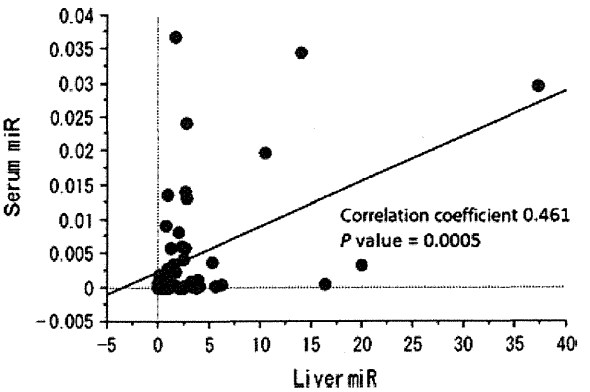

Fig. 1. Correlation between liver and serum miR-122 levels. The serum miR-122 levels were significantly correlated with hepatic miR-122 levels (Spearman correlation coefficient: 0.461; $P = 0.0005$).

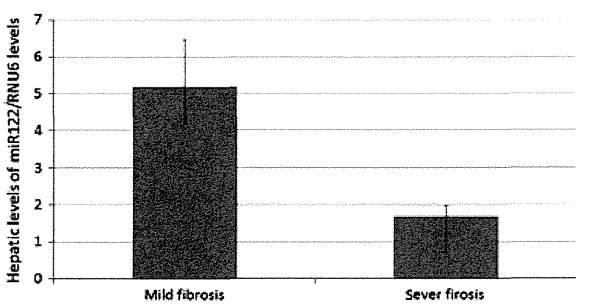

Fig. 2. Correlation between hepatic miR-122 level and the fibrosis stage. Comparisons between groups were performed using the Student's *t*-test ($P = 0.0087$).

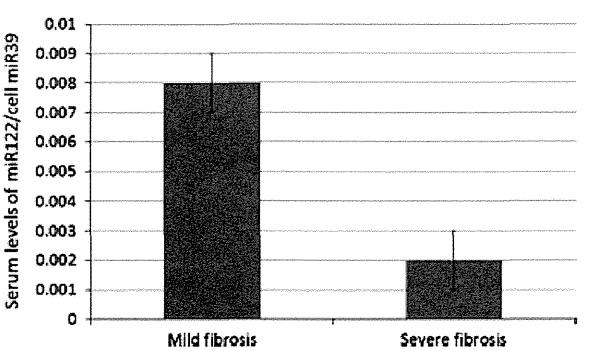

Fig. 3. Correlation between serum miR-122 level and the fibrosis stage. Comparisons between groups were performed using the Student's *t*-test ($P = 0.0191$).

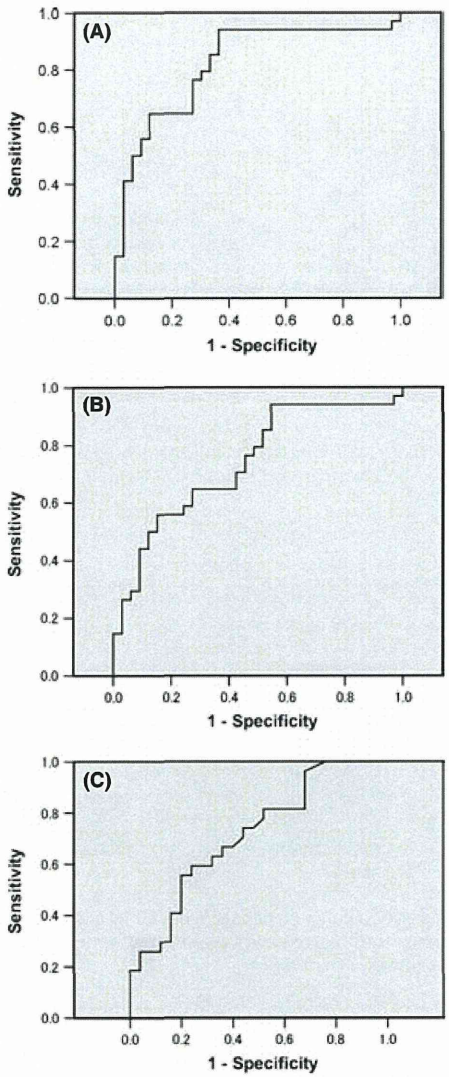

Fig. 4. Receiver operating characteristic (ROC) curve for serum miR-122, hyaluronic acid and Type IV collagen. The area under the ROC curve for serum miR-122 (A), hyaluronic acid (B) and type IV collagen (C) are 0.82, 0.74 and 0.72, respectively.

Discussion

Recent studies have indicated the value of the miR-122 level as a predictive factor of liver disease (27–30). The progression of NAFLD is associated with visceral fat deposition and insulin resistance. miR-122 is a key factor of lipid metabolism (23, 24). In the present study, patients with severe fat deposition showed high miR-122 expression levels in the liver. The role of miR-122 in lipid metabolism has been demonstrated in vitro and in vivo. In in vitro studies using HEP G2 cells, silencing of miR-122 led to the upregulation of the expression of lipid metabolism genes such as fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and sterol binding element binding protein (SREBP), whereas overexpression of miR-122 led to a significant decrease in the levels of these genes (31). In *in vivo* studies, inhibition of miR-122 expression in mice led to the promotion of hepatic fatty acid (FA) oxidation, decreased FA levels, and decreased liver steatosis (23). Thus, these results support our finding that the expression of miR-122 is correlated with liver steatosis.

However, the liver and serum miR-122 levels did not correlate with the NAS and alanine aminotransferase levels. Several recent studies showed that the miR-122 level is associated with liver inflammation (27–29), which was not observed in the present study. However, the previous studies included patients with other liver diseases such as viral hepatitis. In the present study, most of patients had mild inflammation, which may contribute to the lack of a significant difference in miR-122 expression. Moreover, the NAS—established as a scoring system for NAFLD—evaluates not only inflammation but also steatosis. Thus, this discrepancy could be attributed to the different categories of liver disease included in each study.

In the present study, liver miR-122 levels significantly correlated with the liver fibrosis stage. This result is in agreement with those of previous studies, which reported a decrease in liver miR-122 levels at the later stage of fibrosis in patients with liver disease (27–29). Persistent liver injury results in liver cell death, loss of hepatic cells and the accumulation of extracellular matrix. Moreover, the liver miR-122 levels did not correlate with the NAS, which was reflected the inflammation grade of the NAFLD patients. However, hepatocytes are the main source of miR-122. Thus, the progression of liver fibrosis results in the replacement of hepatocytes by extracellular matrix, and thus leads to a decrease in the levels of hepatic miR-122.

Recently, Li *et al.* reported that miR-122 suppressed collagen maturation in hepatic stellate cells and inhibited the proliferation of activated hepatic stellate cells (32). Therefore, decreased miR-122 expression appears to lead to increased collagen maturation and extracellular matrix production, which is consistent with the present results.

In the present study, decreased serum miR-122 levels were detected in association with mild steatosis and advanced fibrosis stage. These results are similar to those noted for hepatic miR-122 expression. Moreover, serum miR-122 expression was well-correlated with hepatic miR-122 expression, which suggests that the miR-122 released from hepatic cells enters into the bloodstream.

The evaluation of liver fibrosis is important to predict the prognosis of patients with NAFLD. Follow-up liver biopsies or repeat liver stiffness assessment is currently necessary to assess liver fibrosis. However, these methods have some limitations. Liver biopsy is an invasive technique and is associated with certain complications

(33, 34). In addition, the utility of liver stiffness measurement is low in obese patients and in those with ascites and hepatic inflammation (35, 36). In the present study, serum miR-122 levels inversely correlated with liver fibrosis, and decreased miR-122 expression was associated with advanced fibrosis stage. Moreover, the ROC curves showed that the ability of the serum miR-122 to predict fibrosis was superior to that of hyaluronic acid and type IV collagen. Therefore, serum miR-122 may be a valuable tool to predict liver fibrosis.

In conclusion, hepatic and serum miR-122 levels are associated with hepatic steatosis and fibrosis, and the serum miR-122 level can serve as a useful predictive marker of liver fibrosis in patients with NAFLD.

Acknowledgements

Conflicts of interest: The authors do not have any disclosures to report.

References

1. Browning JD, Szczepaniak LS, Dobbins R, *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387–95.
2. Madan K, Batra Y, Gupta SD, *et al.* Non-alcoholic fatty liver disease may not be a severe disease at presentation among Asian Indians. *World J Gastroenterol* 2006; **12**: 3400–5.
3. Amarapurkar DN, Hashimoto E, Lesmana LA, *et al.* How common is non-alcoholic fatty liver disease in the Asia-Pacific region and are there local differences? *J Gastroenterol Hepatol* 2007; **22**: 788–93.
4. Chitturi S, Farrell GC, Hashimoto E, *et al.* Non-alcoholic fatty liver disease in the Asia-Pacific region: definitions and overview of proposed guidelines. *J Gastroenterol Hepatol* 2007; **22**: 778–87.
5. Yoshiike N, Lwin H. Epidemiological aspects of obesity and NASH/NAFLD in Japan. *Hepatol Res* 2005; **33**: 77–82.
6. Fan JG, Zhu J, Li XJ, *et al.* Prevalence of and risk factors for fatty liver in a general population of Shanghai, China. *J Hepatol* 2005; **43**: 508–14.
7. Kim HJ, Lee KE, Kim DJ, *et al.* Metabolic significance of nonalcoholic fatty liver disease in nonobese, nondiabetic adults. *Arch Intern Med* 2004; **164**: 2169–75.
8. Itoh S, Yougel T, Kawagoe K. Comparison between nonalcoholic steatohepatitis and alcoholic hepatitis. *Am J Gastroenterol* 1987; **82**: 650–4.
9. Powell EE, Cooksley WG, Hanson R, *et al.* The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990; **11**: 74–80.
10. Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology* 1994; **107**: 1103–9.
11. Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999; **30**: 1356–62.
12. Matteoni CA, Younossi ZM, Gramlich T, *et al.* Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413–9.
13. Shimada M, Hashimoto E, Taniai M, *et al.* Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. *J Hepatol* 2002; **37**: 154–60.
14. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281–97.
15. Ambros V. The functions of animal microRNAs. *Nature* 2004; **431**: 350–5.
16. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 2009; **10**: 126–39.
17. Yuan Y, Zeng ZY, Liu XH, *et al.* MicroRNA-203 inhibits cell proliferation by repressing DeltaNp63 expression in human esophageal squamous cell carcinoma. *BMC Cancer* 2011; **11**: 57.
18. Yang BF, Lu YJ, Wang ZG. MicroRNAs and apoptosis: implications in the molecular therapy of human disease. *Clin Exp Pharmacol Physiol* 2009; **36**: 951–60.
19. Rao DS, O'Connell RM, Chaudhuri AA, *et al.* MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1. *Immunity* 2010; **33**: 48–59.
20. Ortega FJ, Moreno-Navarrete JM, Pardo G, *et al.* MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. *PLoS ONE* 2010; **5**: e9022.
21. Rottiers V, Naar AM. MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol* 2012; **13**: 239–50.
22. Yang YM, Seo SY, Kim TH, Kim SG. Decrease of microRNA-122 causes hepatic insulin resistance by inducing protein tyrosine phosphatase 1B, which is reversed by licorice flavonoid. *Hepatology* 2012; **56**: 2209–20.
23. Esau C, Davis S, Murray SF, *et al.* miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 2006; **3**: 87–98.
24. Elmen J, Lindow M, Silahtaroglu A, *et al.* Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res* 2008; **36**: 1153–62.
25. Schrauder MG, Strick R, Schulz-Wendtland R, *et al.* Circulating micro-RNAs as potential blood-based markers for early stage breast cancer detection. *PLoS ONE* 2012; **7**: e29770.
26. Kleiner DE, Brunt EM, Van Natta M, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313–21.
27. Arataki K, Hayes CN, Akamatsu S, *et al.* Circulating micRoRNA-22 correlates with microRNA-122 and represents viral replication and liver injury in patients with chronic hepatitis B. *J Med Virol* 2013; **85**: 789–98.
28. Trebicka J, Anadol E, Elfimova N, *et al.* Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis. *J Hepatol* 2013; **58**: 234–9.
29. Cermelli S, Ruggieri A, Marrero JA, Ioannou GN, Beretta L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. *PLoS ONE* 2011; **6**: e23937.
30. Qi P, Cheng SQ, Wang H, *et al.* Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. *PLoS ONE* 2011; **6**: e28486.
31. Cheung O, Puri P, Eicken C, *et al.* Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* 2008; **48**: 1810–20.