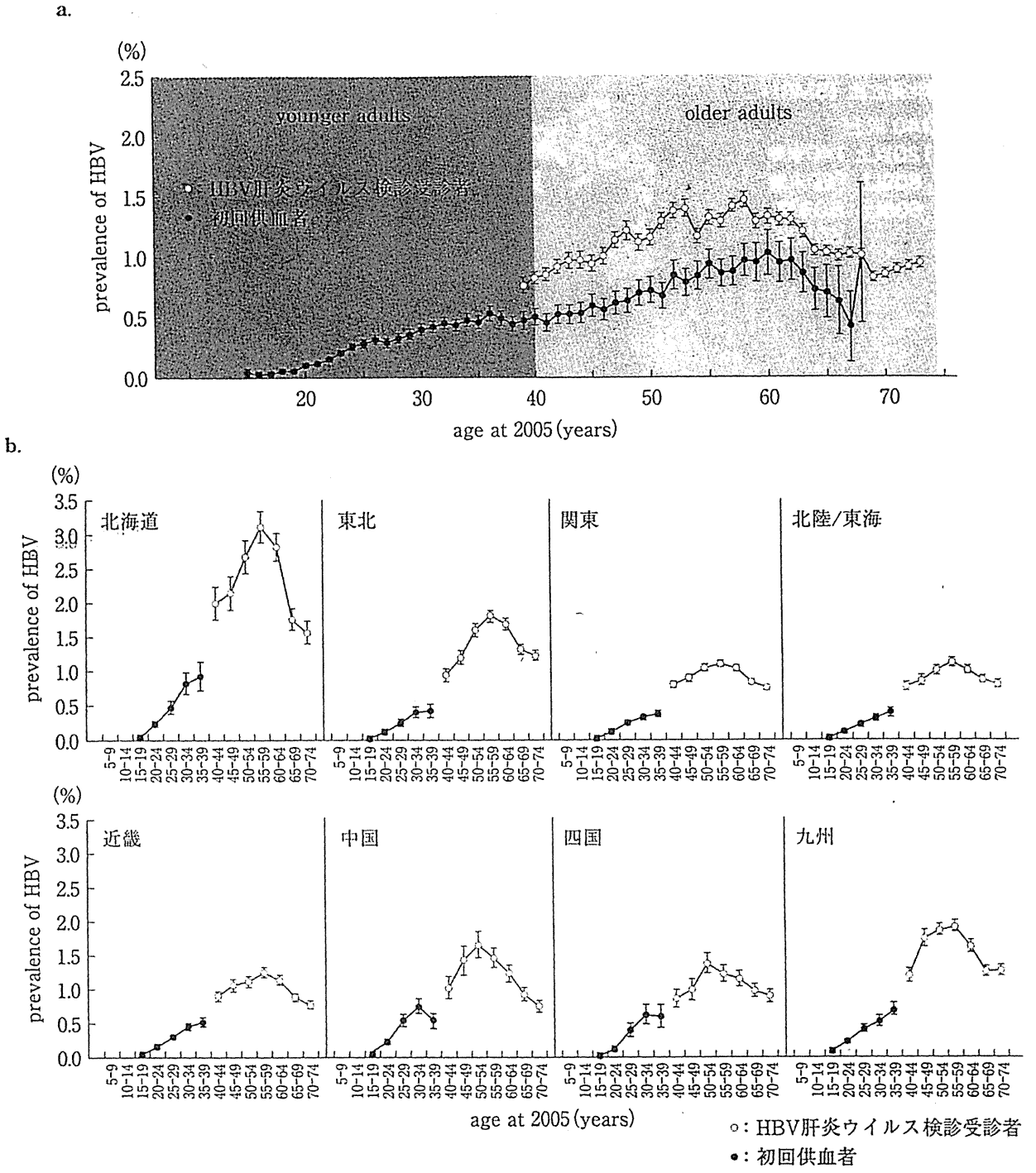


図3-a 初回供血者集団とHBV肝炎ウイルス検診（節目検診）受診者集団からみたHBVキャリア率

-b 8地域別・5歳刻みの年齢階級別にみたHBVキャリア率

を対象とした，保険医療による予防に切り換えられている．

HBV母子感染予防対策実施プロトコールを遵守したHBV母子感染予防が90％以上のキャリア化阻止率で実施されることを仮定すれば，1986年以後出生のコホート集団，すなわち2011年時点25歳以下のコホート集団におけるHBVの持続感染に起因する肝がんは将来激減することが期待される．

表1 HBV母子感染予防実施前・後に出生した年齢集団(1978-94年度)におけるHBs抗原・抗体陽性率の推移(文献[6]より改変)

	出生年	検査数	HBs抗原陽性(%)	HBs抗体陽性(%)
実施前	1978-80	10,437	78(0.75)	159(1.52)
治験期間	1981-85	20,812	46(0.22)	165(0.79)
全面実施	1986-90	32,049	12(0.04)	292(0.91)

*HBs抗体陽性者の中に占めるHBc抗体陽性者の頻度の推移

	出生年	検査数(HBs抗体陽性)	HBc抗体陽性(%)
実施前	1978-80	155	127(81.9)
治験期間	1981-85	157	68(43.3)
全面実施	1986-94	536	59(11.0)

5. 献血を契機に見いだされたHBV DNA陽性，HBs抗原陽性者の特性

日本赤十字血液センターでは輸血用血液に対して，免疫血清学的スクリーニングでは完全には捕捉できないウインドウ期に献血された血液を検出することを目的に，1999年から核酸増幅検査(nucleic acid amplification test: NAT)を導入した．7月から首都圏において試験的に導入後，10月には対象地域を全国に拡大して開始した．2000年2月には，それまでの500人分の血清をプールして検査する方式(500本pool NAT)から，50本pool NAT検査へ，2004年8月には更に20本pool NAT検査への切り換えが行われ，すべての輸血用血液製剤，血漿分画製剤の原料血漿に適用されることで我が国の輸血用血液製剤の安全性は更に高まっている．

NATにより捕捉されたHBV DNA陽性者は，HBV感染の初期ウインドウ期あるいは感染晩期の一時点をとらえていると考えられている[7]．

1999年7月から2007年12月の8年間にNATにより捕捉されたHBV DNA陽性献血者797例についての報告を紹介する[8]．この797例についての年齢別HBV genotype別分布をみると(表2)，我が国で多いとされるgenotype Cは536例(67.3%)であるが，genotype Aは134例(16.8%)であり，20歳代を中心に30歳代，40歳代の男性に見いだされるという特徴がみられている．特に，20-30歳代のHBV DNA陽性者522例では，HBV genotype Aは112例(21.5%)に検出され，そのうち97%(109例)が男性である点が注目される．また，HBV DNA陽性者のうち，HBc抗体陰性であった群におけるHBV genotypeの分布の経年変化をみるとHBV genotype Aの占める比率は，2000年には2.3%であったものが，2003年以降は20%超の比率を占めるようになっている点が注目されている．

また，2006年10月から2007年9月の'1年間の全献血者4,959,541人の内HBs抗原陽性献血者1,979例(HBV genotypeの検討が可能であったのは1,887例)を解析した結果[9]'を紹介すると，HBV genotype Cは62.6%，genotype Bは30.8%，genotype Aは5.6%であり，慢性B型肝炎症例におけるgenotype Aの割合[10,11]とほぼ同等である．一方，1,887例のうちIgM HBc抗体陽性，すなわち感染早期と考えられる61例に占めるgenotype Aの割合は21.7%と高く，急性B型肝炎症例に占める割合に関する報告[11]とほぼ同等であった．

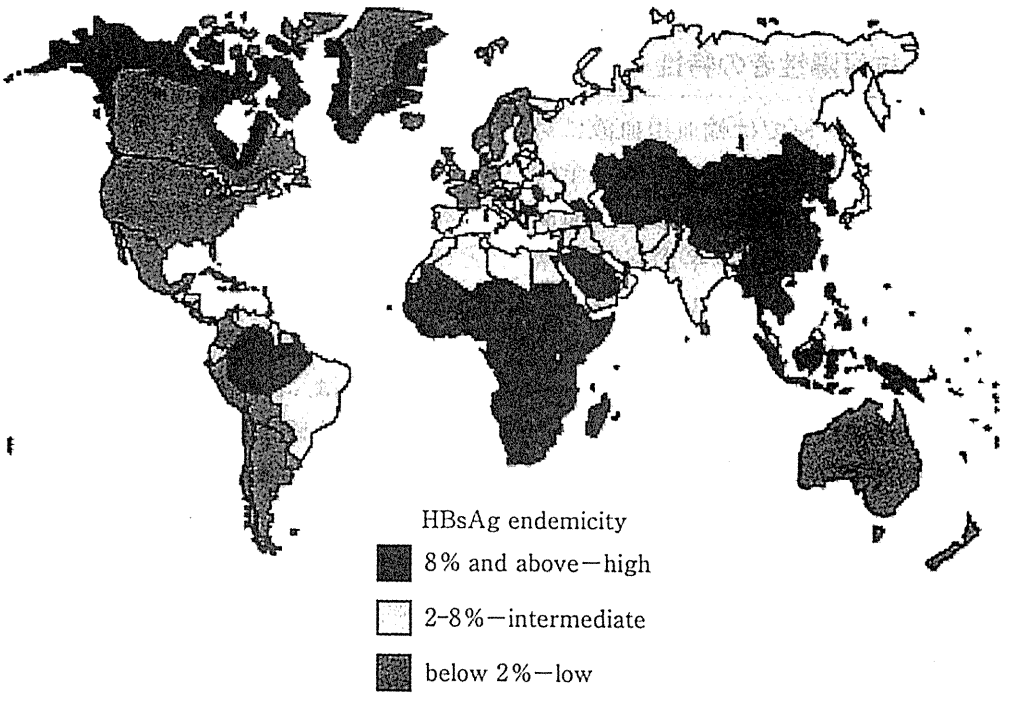
我が国の慢性B型肝炎症例ではHBV genotype CとBが大部分を占めているが，急性B型肝炎症例では，欧米に多いとされるHBV genotype Aによる感染症例の割合が増え，これまでとは異なるルートによる感染が起こっていることが示唆されている．今後，感染予防対策を講じるかどうかについては，一般集団におけるHBV感染の広がりや速度とその大きさ(prevalence, incidence)，genotype別にみたHBVキャリアの自然病態に関する疫学調査が必要とされている．

6. 世界の地域におけるHBVキャリア率

WHO(World Health Organization，2002年)が，一定の手順に従ってまとめた報告・推計[12,13]によると，20億人以上とも考えられる一過性感染を含むHBV感染者のうち3億5千万人は持続感染者であると推定されている．また世界人口の3/4は高度感染地域に居住し，1年間に60万-100万人がB型肝炎に起因する疾病

表2 HBV DNA陽性献血者797例におけるgenotype別にみた分布
(文献[8]より引用)

年齢	genotype													
	A		B		C		D		E		H		計	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
10歳代	4		1	5	19	41	1						25	46
20歳代	68	1	19	18	121	105	1	3		1			209	128
30歳代	41	2	14	4	78	43	2				1		136	49
40歳代	13		15	3	35	12		1			1		64	16
50歳代	5		19	5	34	15	1				1		60	20
60歳代			8	3	28	5							36	8
計	131	3	76	38	315	221	5	4	0	1	3	0	530	267
	134		114		536		9		1		3		797	



From: World Health Organization. Introduction of hepatitis B vaccine into childhood immunization services, 2001, Geneva, WHO, WHO/V&B/01.31

Geographical distribution of chronic hepatitis B virus infection. (Note: The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.)

図4 World distribution map(文献[12]より引用)

(慢性活動性肝炎，肝硬変や肝がん)で死亡していると推定されている．

HBVキャリア率の高い地域は，東南アジア，日本・オーストラリア・ニュージーランドを除く環太平洋地域，サハラ砂漠以南のアフリカ諸国，アマゾン地域，中東・中央アジア，東ヨーロッパの一部の地域である(図4)．これらの地域では，40歳になる前に人口の70-90％が感染し，HBVキャリア率は8-20％と推定されている．

我が国はHBVキャリア率が2-8％の中程度のゾーンに区分されたが，前述のとおり，我が国における疫学的成績からみると2％以下であることは明らかであり，実際よりも高い値に区分されていると考えられる．次回のupdated reportを待ちたい．

おわりに

我が国におけるB型肝炎ウイルス感染をHBs抗原陽性率の地域別年齢別分布からとらえて述べた．

また，目に見える形での効果にはまだ時間がかかると考えられるが，実施から25年経過したHBV母子感染予防対策について紹介した．また，欧米型のHBV感染症例の増加などについても紹介した．

近年，話題になっているHBVユニバーサルワクチネーションについては，多岐にわたる専門家による研究が行われ，導入に対する議論がなされている．社会医学的，疫学的観点からみると，他国とは異なる年齢別HBV感染率やこれまで実施されてきた対策，また，妊婦の受療行動や保険医療制度，検査体制や日本人特有のワクチン施策への反応などを考慮し，我が国特有のHBV感染予防としてのワクチン施策を考える時期に来ていると考えられる．

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ELSEVIER

Optimization of Immunosuppressive Therapy Based on a Multiparametric Mixed Lymphocyte Reaction Assay Reduces Infectious Complications and Mortality in Living Donor Liver Transplant Recipients

Y. Tanaka, H. Tashiro, T. Onoe, K. Ide, K. Ishiyama, and H. Ohdan

ABSTRACT

Aim. We investigated the clinical relevance of immune monitoring by a multiparametric mixed lymphocyte reaction (MLR) assay, wherein the number and phenotype of alloreactive precursors can be quantified by combining the results of carboxyfluorescein diacetate succinimidyl ester labeling and flow cytometry analysis.

Methods. In 51 adult patients undergoing living donor liver transplantation (OLT), immunosuppressive drugs were dosed on the basis of immune monitoring by the MLR assay (optimized protocol: group O). In 64 other patients, the agents were prescribed according to empirical regimens (empirical protocol: group E). In group O, MLR assays were performed at 2- to 4-week intervals until 3 months after OLT and thereafter at 3- to 6-month intervals. Therapeutic adjustments for immunosuppressants were determined by tapering the doses in cases of anti-donor hyporesponsiveness for both $CD4^+$ and $CD8^+$ T-cell subsets.

Results. The 1-year patient and graft survivals in groups O versus E were 90.2% versus 76.6%, respectively. The incidence of acute rejection episodes (ARE) among group O (13.7%) were lower than in cohort E (28.1%). None of the patients in group O while four patients (3%) in group E already have shown chronic rejection to date. The incidences of bacteremia and fungal infections in group O (9.8% and 7.5%, respectively) were lower than in cohort E (18.8% and 12.6%, respectively).

Conclusion. A multiparametric MLR assay may facilitate the development of adequate immunosuppressive regimens.

PATIENTS UNDERGOING LIVER TRANSPLANTATION (OLT) receive immunosuppressants according to empirical protocols, which seek to take into account the risks of under- versus oversuppression, rejection, infection, and adverse drug reactions. However, an individually optimized immunosuppressive protocol developed on the basis of immune monitoring would be useful to avoid these undesirable effects. The aim of this study was to investigate the clinical relevance of immune monitoring by a multiparametric mixed lymphocyte reaction (MLR) assay, wherein the number and phenotype of alloreactive precursors is quantified by combining the results of carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling with flow cytometry (FCM) analysis.

PATIENTS AND METHODS

Patient Population and Immunosuppressive Protocol

We enrolled 115 patients who underwent adult-to-adult living donor OLT (LDLT). The basic immunosuppressive regimen consisted of tacrolimus or cyclosporine with methylprednisolone in gradually tapered doses. Among 64/115 patients, the immunosuppressants were dosed according to empirical regimens (empirical protocol: group E). In 51 other patients, immunosuppressive drugs were prescribed on the basis of immune monitoring by the MLR assay (optimized protocol: group O). In this group, MLR assays were performed at 2- to 4-week intervals until 3 months after LDLT, and thereafter at intervals of 3 to 6 months.

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MLR Assay

Peripheral blood mononuclear cells (PBMCs) prepared from recipients (autologous controls), donors or healthy volunteers (third-party control) serving as stimulator cells were irradiated with 30 Gy. Recipient responder cells were labeled with CFSE. Both the stimulator and responder cells were cocultured at 37°C in the dark for 5 days, as described previously.[1] After MLR culture, harvested nonadherent cells were stained with either phycoerythrin-conjugated CD4 or CD8 monoclonal antibodies (mAbs; BD Pharmingen, San Diego, Calif, USA) together with allophycocyanin-conjugated CD25 mAb (BD Pharmingen). The four-color FCM was performed on a FACSCalibur dual-laser cytometer (Becton Dickinson, Mountain View, Calif, USA). Dead cells were excluded from the analysis by light scatter and/or propidium iodide fluorescence.

Quantifying Proliferation of $CD4^+$ and $CD8^+$ T Cells

Precursor frequency (PF), proliferation index (PI), and stimulation index (SI) were quantitatively estimated using the previously described method.[1]

In brief, divisions of reactive T cells, which were identified by their CFSE intensities, labeled from 0 to n were the dividing time. A single cell dividing n times generates 2^n daughter cells. Using this mathematical relationship, the number of division precursors was extrapolated from the number of daughter cells of each division and from proliferation events and PF in $CD4^+$ and $CD8^+$ T-cell subsets. Using these values, proliferation events and PIs were calculated. The SI was calculated by dividing the PIs of allogeneic combinations by those of autologous controls.

Adjustments for Immunosuppressants

On the basis of the proliferation analysis of $CD4^+$ and $CD8^+$ T-cell subsets in response to anti-donor versus anti-third party stimuli in MLR, we categorized the immune status as hypo-, normo-, or hyperresponsive (Fig 1). Therapeutic adjustments for immunosuppressants were determined by tapering dosages in cases exhibiting anti-donor hyporesponsiveness in both T-cell subsets or by increasing them for anti-donor hyperresponsiveness.

Statistical Analysis

For continuous variables, parametric analyses were performed using Student t test, and the Mann-Whitney U test for nonparametric analyses. Categorical variables and postoperative courses were compared using χ^2 tests with Yates correction. For factors determined to be significant for survival rates, as well as incidences of infection and ARE upon univariate analysis, we performed multivariate analyses using logistic regression. A difference was considered significant if the

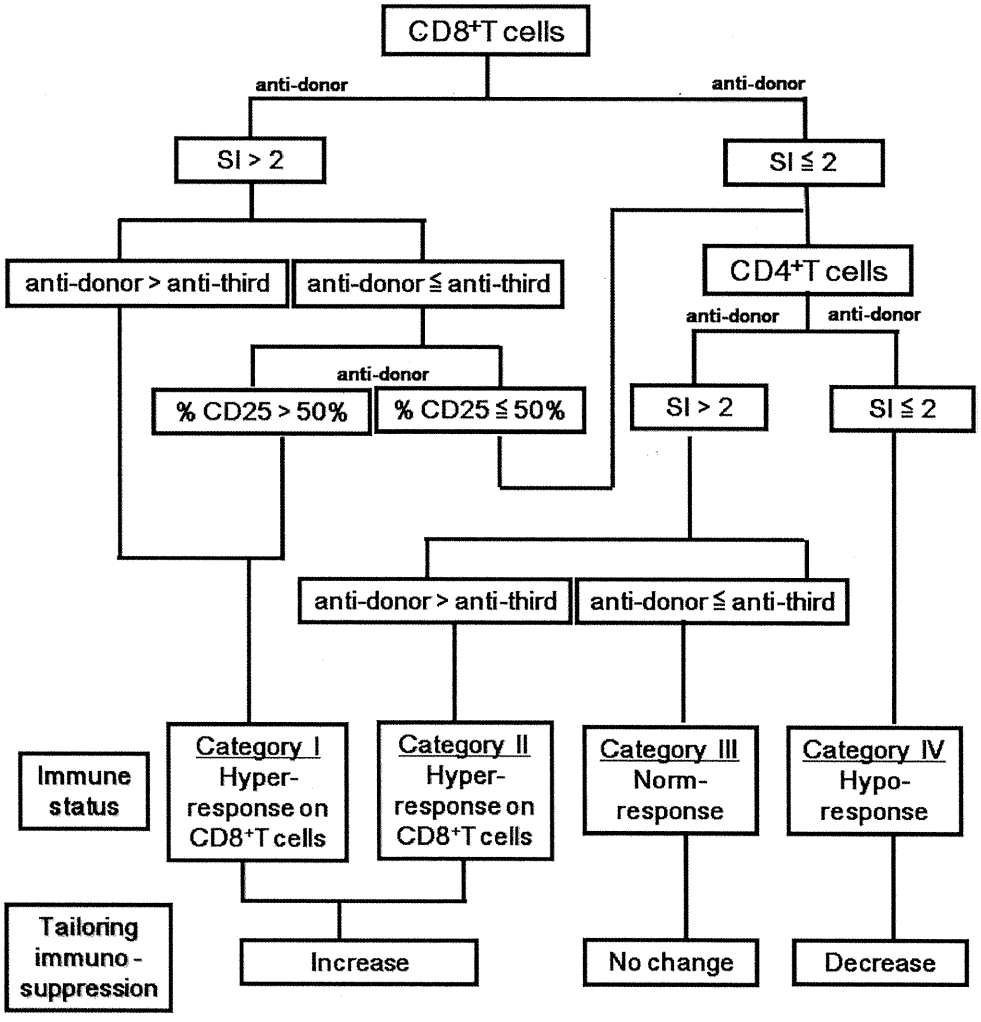

Fig 1. Algorithm to determine anti-donor alloreactivity in liver transplant patients. The immune status of liver transplantation patients was classified into four categories. By analyzing the proliferation and CD25 expression for the $CD4^+$ and $CD8^+$ T-cell subsets in response to anti-donor and anti-third party stimuli, the immune status was categorized as hypo-, normo-, or hyperresponsive. In patients with hyperresponsive immune status on either $CD4^+$ or $CD8^+$ T cells, immunosuppressants were increased. In patients with normoresponsive immune status, immunosuppressant tapering was abandoned. Only in patients with hyporesponsive immune status, immunosuppressant therapy was tapered off. SI, stimulation index.

Table 1. Patient Profile

	Emprical Group (group E)	Optimized Group (group O)	*P* Value
Case number	64	51	
Age	51.2 ± 10.7	54.1 ± 9.4	.12
Sex (M vs F)	35 vs 29	21 vs 30	.23
MELD score	19.2 ± 9.1	15.4 ± 6.4	.002
Donor aging	35.0 ± 12.4	37.1 ± 12.5	.36
Relationship of donor and recipient (blood relationship vs non-blood relationship)	11 vs 53	7 vs 54	.61
Child classification (A vs B vs C)	8 vs 21 vs 35	11 vs 17 vs 23	.38
GRWR	0.99 ± 0.25	0.97 ± 0.27	.72
Operation time (min)	709.7 ± 113.2	750.2 ± 139.8	.09
Blood loss during surgery (mL)	4501.5 ± 3596.0	4416.3 ± 2703.6	.89
Original disease (acute liver failue vs chronic liver failue)	7 vs 57	1 vs 50	.06

MELD, Model for End-stage Liver Disease; GRWR, graft-recipient body weight ratio.

P value was less than .05. Statistical analyses were performed using the SPSS statistical software version 16 (Chicago, Ill, USA).

RESULTS

The patient profiles are shown in Table 1. The Model for End-stage Liver Disease score in group E was higher than that in group O, probably reflecting the greater proportion of patients with acute liver failure as an original disease in group E compared with group O. There were no differences in other background factors between the groups. Figure 2 shows the change in immune status determined by the MLR assay during the observation period. The proportion of patients showing anti-donor hyporesponsiveness gradually increased over time. At 3 to 6 months after transplantation, immunosuppressants were reduced in approximately 60% of group O patients. We also investigated the incidence of infectious disease within 1 year after OLT. In group O, the incidences of sepsis and fungal, bacterial, or cytomegalovirus infection were lower than those in group E, although the difference did not reach significance ($0.05 < P < 0.1$, Fisher exact test; Fig 3A). The incidence of ARE in the O cohort was also lower than that in group E (Fig 3B). Furthermore, none of the patients in group O showed chronic rejection versus 3% in group E (Fig 3B). The 6- and 12-month patient survival rates in group O were a bit higher than those in group E, although the difference did not reach significance (Fig 3C). Univariate analyses of differences in the incidences of ARE and infection, as well as 6- and 12-month survival rates between the groups are shown in Table 2. Multivariate analyses to determine factors associated with these metrics showed only donor age to be significantly associated with 1-year survival rates (Table 3).

An additional benefit of optimizing immunosuppression under the regimen of immune monitoring would be a

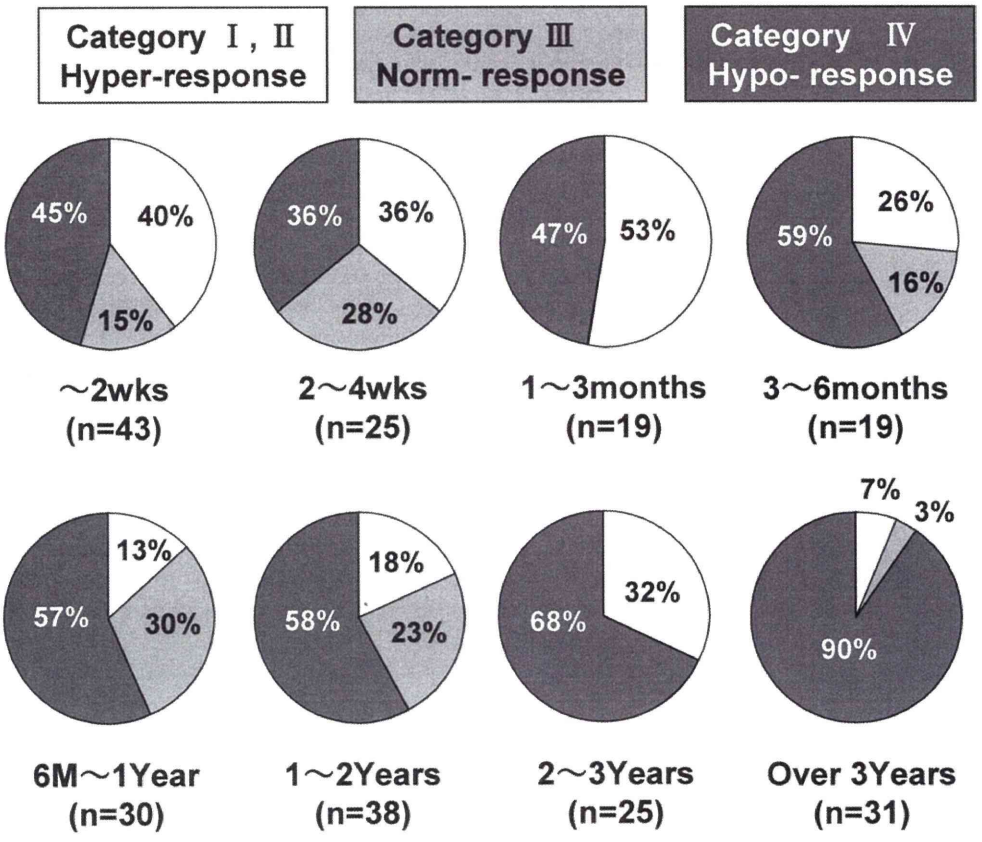

Fig 2. Change in immune status after liver transplantation. The immune status of liver transplantation patients was classified into four categories. By analyzing the proliferation and CD25 expression for the $CD4^+$ and $CD8^+$ T-cell subsets in response to anti-donor and anti-third-party stimuli, the immune status was categorizedas hypo-, normo-, or hyperresponsive. The proportions of patients categorized into each immune status at various times after liver transplantation are shown.

successful response to vaccination to prevent viral infections. For instance, hepatitis B virus (HBV) vaccination can prevent reinfection with HBV. However, the immunosuppressive environment is believed to result in a poor response to vaccination.[2] We observed that the overall response rate to HBV vaccination among group O was higher than cohort E, suggesting that minimized immunosuppression may enable posttransplant HBV vaccination, a promising prophylactic strategy (Fig 4).

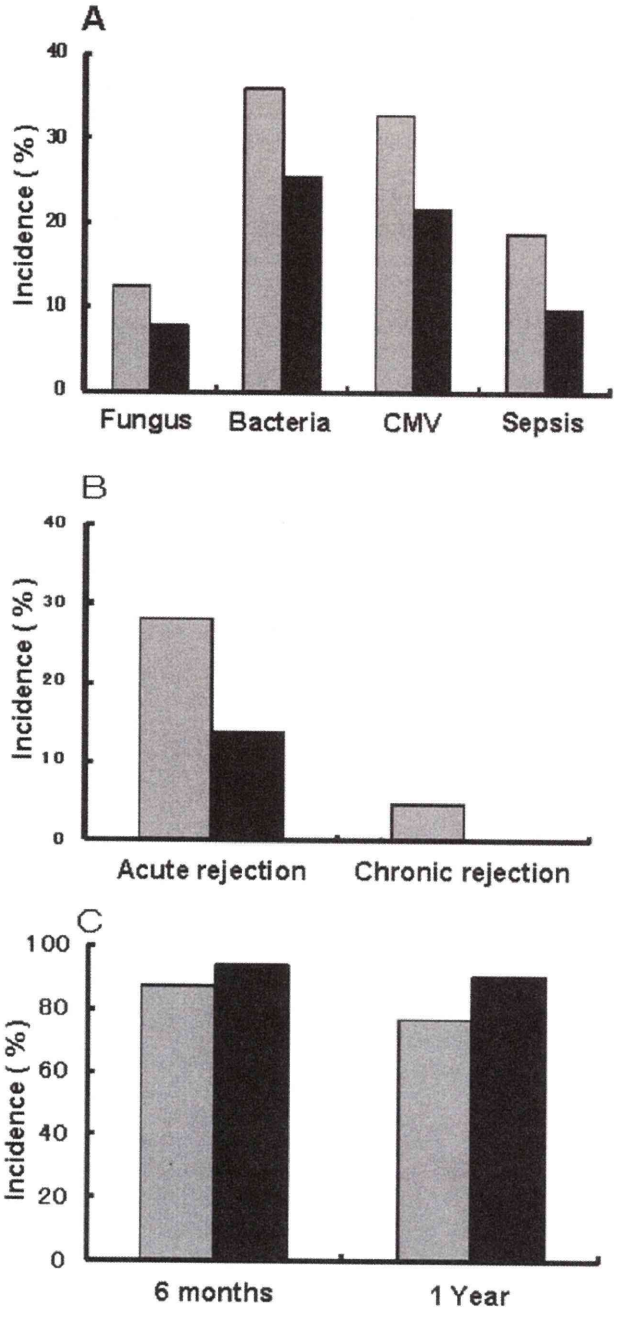

Fig 3. Incidences of infectious disease, acute rejection, and chronic rejection within 1 year after liver transplantation. The incidences of infectious disease **(A)**, acute rejection, and chronic rejection **(B)** within 1 y after liver transplantation were investigated. In addition, the 6-month and 1-year patient survival rates in each group are shown **(C)**. CMV, cytomegalovirus.

Table 2. Results of Univariate Analyses to Determine the Difference in the Incidence of Rejection and Infection and 6-mo and 1-y Survival Between Empirical Group and Optimized Group

	P
Incidence of acute rejection	.055
Incidence of infection	.776
6-mo survival	.231
1-y survival	.107

DISCUSSION

Anti-donor alloreactivity, defined as the number and phenotype of alloreactive precursors in the recipient, can be used to monitor graft rejection, to guide treatment to reduce ARE, or to withdraw immunosuppression. MLR using PBMCs is widely used in both experimental and clinical transplantation to evaluate T-cell responses to allogeneic stimulation. However, conventional assays of MLR using tritiated thymidine incorporation show little predictive value because of their low level of reproducibility.[3] This limitation might be caused—at least in part—by the presence of nonviable cells (which might include unexpectedly surviving stimulator cells) that retain the ability to incorporate tritiated thymidine. With a CFSE-based method, the proliferation of viable $CD4^+$ and $CD8^+$ responder T cells in response to allostimulation is quantified separately using a multiparameter FCM. A lack of proliferation by both $CD4^+$ and $CD8^+$ T cells in anti-donor MLR may reflect effective suppression of the anti-donor responses. When remarkable proliferation was observed among $CD4^+$ but not in $CD8^+$ T cells, we did not observe cytotoxic activity against donor cells in subsequent cell-mediated lympholysis assays in our previous studies.[4] In contrast, remarkable proliferation of $CD8^+$ T cells reflect strong anti-donor responses. We further examined CD25 expression on the proliferating $CD8^+$ T cells by multicolor FCM. In our previous studies, a remarkable elevation of CD25 expression on proliferating $CD8^+$ T cells was observed to reflect their cytotoxic activity toward donor cells.[4] By using such a multiparametric MLR assay, we demonstrated that a careful evaluation of recipient immune status facilitated the development of adequate immunosuppressive regimens. Optimization of immunosuppression in this manner seems to be a promising strategy to reduce infectious complications and mortality among patients undergoing LDLT.

Table 3. Multivariate Analysis of 1-y Survival

Variable	Hazard Ratio	95% Confidence Interval	*P* Value
Donor age	0.959	0.923–0.997	.033

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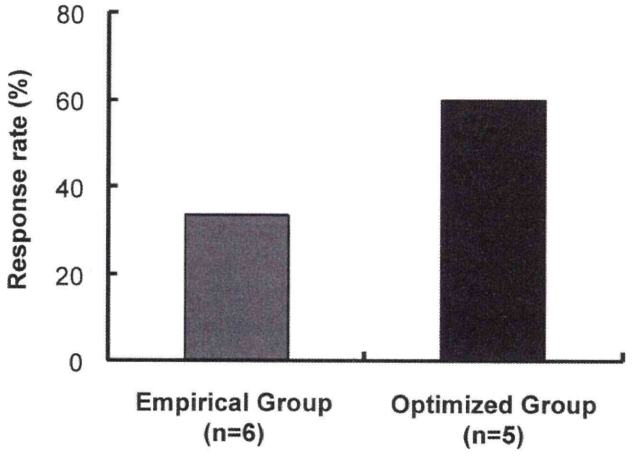

Fig 4. Overall response rate to hepatitis B virus (HBV) vaccination within 1 year after commencing HBV vaccination. All participants received a yeast-derived recombinant adsorbed HBV vaccine (Bimmugen) subcutaneously every 4 weeks at a dose of 10 to 20 μg (0.5–1.0 mL) in combination with HBIg and lamivudine/adefovir. HBIg immunoprophylaxis was continued during primary immunization (dose, 1000–2000 IU every 4 weeks). The response to vaccination was defined as (1) a confirmed increase in the anti-hepatitis B surface (HBs) antigen HBs titer to >100 IU/L that could not be explained by HBIg administration and (2) sustained anti-HBs titer to >100 IU/L after discontinuation of combined administration of the vaccine and HBIg. If the anti-HBs titer exceeded the responsive increasing level, HBIg substitution and vaccine administration were discontinued. Lamivudine/adefovir prophylaxis was additionally discontinued, if the anti-HBs titer was maintained effectively without HBIg administration. The vaccine was continuously and indefinitely administered till acquired immunity was elicited.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Impact of Pegylated Interferon Therapy on Outcomes of Patients with Hepatitis C Virus-Related Hepatocellular Carcinoma After Curative Hepatic Resection

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ABSTRACT

Background. Several published reports investigating the effects of interferon (IFN) therapy on survival and tumor recurrence after curative resection of hepatocellular carcinoma (HCC) have been inconclusive. The aim of this study is to investigate the efficacy of pegylated-IFN (peg-IFN) therapy after curative hepatic resection for HCC in patients infected with hepatitis C virus (HCV).

Methods. Data from 175 patients who underwent curative hepatic resection for HCC associated with HCV were retrospectively collected and analyzed; 75 patients received peg-IFN therapy after surgery, whereas 100 patients did not receive IFN therapy. To overcome biases resulting from the different distribution of covariates in the two groups, a one-to-one match was created using propensity score analysis. After matching, patient outcomes were analyzed.

Results. After one-to-one matching, patients ($n = 38$) who received peg-IFN therapy after surgery and patients ($n = 38$) who did not receive IFN therapy had the same preoperative and operative characteristics. The 3- and 5-year overall survival rates of patients who received peg-IFN therapy after hepatic resection were significantly higher than those of patients who did not receive IFN therapy ($P = 0.00135$). The 3- and 5-year overall survival rates were 100 and 91.7% and 76.6 and 50.6% in the peg-IFN group and non-IFN group, respectively. There was no significant difference in disease-free survival between the two matched groups ($P = 0.886$).

Conclusion. Peg-IFN therapy may be effective as an adjuvant chemopreventive agent after hepatic resection in patients with HCV-related HCC.

Hepatic resection is a well-accepted therapy for hepatocellular carcinoma (HCC), but many patients show cancer recurrence and the cumulative 5-year HCC recurrence rate exceeds 70%.[1–3] This high incidence of tumor recurrence after hepatic resection remains a major drawback. Some benefits of interferon (IFN) therapy on tumor recurrence and survival have been reported.[4–10] IFN suppresses replication of hepatitis C virus (HCV) and exerts a tumoricidal effect on a number of tumors, including HCC.[10,11] However, several randomized controlled trials (RCTs) have revealed inconclusive results regarding the effects of IFN on survival and tumor recurrence after curative resection or ablation of HCC, either because the effects were not statistically significant or because they were considered only with respect to defined subpopulations.[12–15]

Recently, combination therapy consisting of pegylated interferon (peg-IFN) plus ribavirin (RBV) has been developed, and the effect of this combination has been reported to be higher than that of conventional IFN therapy.[16,17] Peg-IFN has an extended serum half-life that provides viral suppression for 7 days, thus allowing weekly administration and enhanced clinical efficacy.[17] Most Japanese patients infected with HCV are infected with HCV genotype Ib and have high viral load. Moreover, treatment with conventional IFN is complicated by a low sustained viral response (SVR) rate of 20–30%.[18–20]

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However, peg-IFN plus RBV combination therapy has good tolerability in Japanese patients with HCV and resulted in an SVR rate of approximately 40–50%.[21–23] The impact of adjuvant immunotherapy with IFN after curative resection of HCC is debatable, and few studies have investigated the effects of peg-IFN plus RBV combination therapy on survival and recurrence after curative resection of HCC.

In the present study, we aim to investigate the impact of peg-IFN plus RBV combination therapy on survival and HCC recurrence after curative resection in patients infected with HCV.

PATIENTS AND METHODS

Patients and HCV Diagnosis

From June 2003 to June 2009, 370 HCC patients underwent hepatectomy as initial treatment at the Department of Gastroenterological Surgery, Hiroshima University Hospital, Japan. Of the 370 patients, 175 patients who were HCV RNA-positive/hepatitis B surface antigen-negative underwent curative hepatectomy. Of the 175 patients, 75 patients received IFN therapy after hepatectomy, and 100 patients did not receive any IFN therapy. Of the 75 patients who received IFN, 20 patients who received IFNs such as IFN-α or IFN-β were excluded. Of the 55 patients who received peg-IFN therapy, 43 patients who started peg-IFN within 9 months after curative resection were enrolled in this analysis. Twenty-four patients who had early recurrence of HCC within 9 months after surgery were excluded from the 100 patients who did not receive any IFN therapy, because these patients could lose the opportunity to receive IFN therapy for HCC recurrence if these patients were assigned to the peg-IFN therapy. Consequently, 119 patients were eventually enrolled in this study. Of these 119 patients, 43 received peg-IFN therapy within 9 months after hepatectomy, and 76 did not receive any IFN therapy.

Curative hepatectomy was defined as removal of all recognizable tumors. HCV RNA levels were measured by quantitative reverse-transcription polymerase chain reaction (RT-PCR; Amplicor, Roche Diagnostic Systems, CA, USA). HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of the NS5 region. HCV negativity was evaluated by quantitative RT-PCR. The lower limit of the assay was 5 kIU/ml (equivalent to 5,000 copies/ml) in the quantitative method and 50 IU/ml (equivalent to 50 copies/ml) in the qualitative method. SVR was defined as undetectable HCV RNA at 24 weeks after completion of IFN therapy. The study was approved by the concerned institutional review boards. Written informed consent was obtained from all patients.

Preoperative Diagnosis and Evaluation of HCC

Hepatocellular carcinoma was diagnosed on the basis of routine imaging modalities such as Doppler ultrasonography (US), computed tomography (CT) during hepatic angiography (CTHA) and CT during arterial portography (CTAP), and magnetic resonance imaging. Tumor stage, liver damage classification, and surgical procedures were defined according to the General Rules for Clinical and Pathologic Study of Primary Liver Cancer, fifth edition, by the Liver Cancer Study Group of Japan.[24]

Hepatectomy

The surgical procedure was determined according to tumor extent and hepatic reserve function. Liver function was assessed by liver damage classification, Child–Pugh classification, and indocyanine green retention rate at 15 min (ICGR 15).[25,26] If permitted by liver function, anatomic resection was performed.[27,28] In patients with insufficient hepatic reserve, limited resection was performed. We divided the liver parenchyma by using an ultrasonic dissector.[29] Postoperative complications were graded according to the method described by Clavien et al.[30]

Follow-Up

Follow-up evaluation after the surgery consisted of monthly blood chemistry tests and measurements of levels of tumor markers, including alpha-fetoprotein and des-gamma-carboxy prothrombin. Patients were examined by US every 3 months and by CT every 6 months. When recurrence was indicated by any of these examinations, patients were examined by CTAP and CTHA.

Patient Selection for IFN Therapy

Patients with HCV genotype 1b in the IFN group received peg-IFNα-2b (Pegintron; Schering-Plough, NJ, USA) at weekly dosage of 1.5 μg/kg subcutaneously for 48 weeks. Daily RBV (Rebetrol, Schering-Plough) was administered orally for 48 weeks, and the dosage was adjusted according to weight (600 mg for patients weighing $\leq$60 kg, 800 mg for those weighing 60–80 kg). Patients with HCV genotype 2 received IFN monotherapy for 24 weeks. Blood samples were obtained every 4 weeks and analyzed for HCV RNA levels. All patients were informed about IFN therapy after hepatectomy, and only consenting patients received IFN therapy. The eligibility criteria for IFN therapy were as follows: (1) detectable serum HCV RNA level, (2) Eastern Cooperative Oncology

Group (ECOG) performance score of 0 or 1, (3) platelet count ≥70,000/μl, (4) patients with no uncompensated cirrhosis (Child class C), and (5) hemoglobin concentration ≥10 g/dl. Peg-IFN therapy was commenced within 24 weeks of surgery or after the eligibility criteria were fulfilled.

Safety Assessments and Dose Modification of Peg-IFN Therapy

Adverse events were graded as mild, moderate, severe, or potentially life-threatening according to a modified World Health Organization grading system. The dose of peg-IFN was decreased by 50% and that of RBV was lowered to half in case of severe adverse events or when laboratory results revealed any of the following: hemoglobin concentration <10 g/dl in patients with no cardiac disease, decrease in hemoglobin concentration >2 g/dl in patients with cardiac disease, white blood cell count <3,000/mm^3, or platelet count <50,000/mm^3. Full dosage could be resumed on resolution of the adverse events. Treatment was permanently discontinued in case of life-threatening events or when laboratory results revealed hemoglobin concentration <7.5 g/dl after 4 weeks of dose reduction, white blood cell count <1,500/mm^3, or platelet count <30,000 mm^3.

Treatment for Recurrence

Patients with intrahepatic HCC recurrence were managed with ablative therapies such as radiofrequency ablation (RFA), percutaneous ethanol injection therapy, transarterial chemoembolization, or surgery including living-donor liver transplantation according to the tumor characteristics (number, size, and location of the tumors) and liver function.

Statistical Analyses

Categorical variables were compared using the chi-square test, and continuous variables were compared using the Mann–Whitney *U*-test. Overall survival and disease-free survival analyses were performed using Kaplan–Meier methods; comparisons between different groups were performed using the log-rank test. *P* value of less than 0.05 was considered significant. Calculations were performed using SPSS software (version 16; SPSS Inc., IL, USA).

Propensity analysis was performed using logistic regression to create a propensity score for the IFN and non-IFN therapy groups.[31,32] Variables entered in the propensity model were age, sex, HCV genotype, liver function test, tumor factors, and operative factors. The model was then used to provide a one-to-one match between the two groups by using the nearest-neighbor matching method.[33,34] Survival and disease-free survival analyses were performed in each matched subgroup to assess the impact of peg-IFN therapy on mortality after adjusting for the confounding factors.

RESULTS

Characteristics and Postoperative Course of the Entire Population

Differences in the characteristics of patients who received peg-IFN therapy after hepatic resection and those who did not receive IFN therapy after hepatic resection are presented in Table 1. Patients who received peg-IFN therapy were younger (65 vs. 71 years; $P = 0.0003$). Regarding tumor characteristics, there was no significant difference between the two groups. Operation times tended to be longer in patients who received peg-IFN therapy than in those who did not receive IFN therapy (260 vs. 242 min; $P = 0.05$). There were no hospital-related deaths in this study. Postoperative complications did not differ between the two groups. In the entire population, the 3- and 5-year overall survival rates of patients who received peg-IFN therapy after hepatic resection were significantly higher than those of patients who did not receive IFN therapy ($P = 0.0024$) (Fig. 1a). However, there was no significant difference in disease-free survival between the two groups ($P = 0.795$) (Fig. 1b).

Results After Propensity Score Matching

Characteristics of the patients after propensity score analysis are presented in Table 1. Thirty-eight of the 43 patients who received peg-IFN therapy after hepatic resection and an equal number of the 76 patients who did not receive IFN therapy were matched after covariate adjustment. The study group of 76 patients was well matched; in particular, all covariates that significantly affected recurrence and postoperative liver failure in the entire study group were equally distributed between the two matched groups. Matched patients who received peg-IFN therapy after hepatic resection had similar total bilirubin and serum albumin levels and similar platelet counts to matched patients who did not receive IFN therapy. Similarly, the tumor characteristics, the surgical procedure, operation times, and blood loss during the operation in matched patients who received peg-IFN therapy were almost similar to those in patients who did not receive IFN therapy. There were no hospital-related deaths in the matched groups. Postoperative complications also did not differ between the two groups. The median follow-up period for patients who received peg-IFN and those who

TABLE 1 Baseline characteristics and operative data on patients who underwent hepatectomy: data are reported for whole study and for the matched study population after propensity score analysis

	Overall series		*P* value	Propensity-matched series		*P* value
	IFN (+) $n = 43$	IFN (−) $n = 76$		Peg-IFN (+) $n = 38$	IFN (−) $n = 38$	
Age (years)	65 (53–78)	71 (48–83)	0.0003	65.5 (53–75)	69 (51–80)	0.2
Sex (male/female)	27/16	47/29	0.918	23/15	25/13	0.634
Preoperative IFN	24 (55.8%)	29 (38.1%)	0.06	20 (52.6%)	14 (36.8%)	0.16
HCV genotype			0.876			0.6
1b	34	61		29	27	
2b	9	15		9	11	
Diabetes mellitus	11 (25.6%)	22 (28.9%)	0.856	11 (28.9%)	13 (34.2%)	0.621
ECOG PS			0.831			0.644
0	39	68		36	35	
1	4	8		2	3	
Platelet (104/mm^3)	10.3 (3.3–26.6)	10.3 (3.8–40.3)	0.381	9.75 (3.3–21.5)	11.2 (3.8–40.3)	0.454
T-Bil (mg/dl)	0.7 (0.3–1.4)	0.8 (0.3–1.7)	0.292	0.7 (0.4–1.4)	0.7 (0.3–1.7)	0.798
AST (IU/l)	42 (18–121)	48 (16–150)	0.152	43.5 (18–127)	41.5 (6–150)	0.567
ALT (IU/l)	38 (13–127)	41.5 (10–196)	0.987	40.5 (11–127)	37.5 (10–196)	0.226
Albumin (g/dl)	3.8 (2.8–5.2)	3.8 (2.5–4.9)	0.215	3.8 (2.8–5.2)	3.8 (2.5–4.5)	0.469
ICGR 15 (%)	17.9 (7.4–77.4)	18.7 (4.6–50.5)	0.734	17.65 (7.4–40.0)	17.55 (4.6–40.0)	0.561
AFP (ng/ml)	11.6 (0.5–3405)	27.6 (0.5–36572)	0.176	13.95 (0.5–3405)	22.9 (0.5–513)	0.635
Child–Pugh grade			0.665			0.556
A	41 (95.3%)	69 (90.8%)		37 (97.4%)	36 (94.7%)	
B	2 (4.7%)	7 (9.2%)		1 (2.6%)	2 (5.3%)	
Hepatic resection			0.322			0.373
Hr0	20 (46.5%)	49 (64.5%)		18 (47.4%)	23 (60.5%)	
HrS	13 (30.2%)	18 (23.7%)		12 (31.6%)	9 (23.7%)	
Hr1	3 (7.0%)	4 (5.3%)		2 (5.3%)	3 (7.9%)	
Hr2	7 (16.3%)	5 (6.6%)		6 (15.8%)	2 (5.3%)	
Hr3	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
Operation time (min)	260 (128–623)	242 (90–580)	0.0514	257 (128–623)	247.5 (90–580)	0.18
Blood loss (ml)	200 (20–1900)	225 (10–960)	0.996	210 (20–1900)	210 (10–960)	0.803
Postoperative complications			0.933			0.798
IIIa	4	6		2	2	
IIIb	1	1		1	1	
IVa	1	1		1	0	
Stage			0.315			0.293
I	14 (32.6%)	19 (25.0%)		13 (34.2%)	9 (23.7%)	
II	18 (41.9%)	44 (57.9%)		15 (39.5%)	23 (60.5%)	
III	9 (20.9%)	12 (15.8%)		9 (23.7%)	6 (15.8%)	
IV-A	2 (4.7%)	1 (1.3%)		1 (2.6%)	0 (0.0%)	
Single tumor	28 (65.1%)	57 (75.0%)	0.252	25 (65.8%)	29 (76.3%)	0.312
Tumor size			0.712			0.589
≥3 cm	15 (34.9%)	24 (31.6%)		10 (26.3%)	8 (21.1%)	
<3 cm	28 (65.1%)	52 (68.4%)		28 (73.7%)	30 (78.9%)	
Vascular invasion	4 (9.3%)	3 (3.9%)	0.233	3 (7.9%)	0 (0.0%)	0.239

Continuous variables expressed as median (range)

Hepatic resection and stage were according to General Rules for the Clinical and pathological Study of Primary Liver Cancer, by Liver cancer Study Group of Japan, 5th edition, Kanehara Co., Ltd

Hr0: limited resection, HrS: segmentectomy, Hr1: sectionectomy, Hr2: hemihepatectomy, Hr3: more than hemihepatectomy

T-Bil total bilirubin, *PS* performance status, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ICGR 15* indocyanine green retention rate at 15 min, *AFP* alpha-fetoprotein,

did not receive IFN therapy was 3.8 (1.2–6.9) and 3.5 (1.3–6.8) years, respectively. In the matched study groups, the 3- and 5-year overall survival rates of patients who received peg-IFN therapy after hepatic resection were significantly higher than those of patients who did not receive IFN therapy ($P = 0.00135$) (Fig. 1c). However, there was no significant difference in disease-free survival between the two matched groups ($P = 0.886$) (Fig. 1d).

In the matched 38 patients of the peg-IFN group, peg-IFN therapy was initiated at a median of 4.3 (0.9–9.6) months after hepatic resection. Thirty-one of 38 HCC patients began peg-IFN therapy within 6 months after hepatectomy. Seven patients required more than 6 months to commence peg-IFN therapy. Two patients required a longer time to recover platelet counts of more than 70,000/μl. Five patients required a longer time to decide to receive peg-IFN therapy. Sixteen (42.1%) of the matched 38 patients who received peg-IFN therapy after hepatectomy attained SVR. Among 16 patients who attained SVR, 10 patients received full-dose peg-IFN therapy without dose reduction, whereas 6 patients received a reduced dose of peg-IFN and/or RBV until completion of treatment. Nine patients discontinued peg-IFN therapy because of adverse events such as thrombocytopenia and neutropenia ($n = 2$), skin eruption ($n = 1$), depression ($n = 2$), and severe malaise ($n = 4$). Three patients discontinued peg-IFN therapy because of HCC recurrence. Adherence to peg-IFN therapy was 68.4% in this study. No life-threatening adverse events were observed, and none of the total 15 deaths in both sets of matched patients were related to the IFN treatment or to surgical procedures. The 3- and 5-year overall survival rates of patients ($n = 16$) who attained SVR after peg-IFN therapy were 100% and 100%, respectively; those of patients who did not attain SVR ($n = 22$) were 100 and 85.7%, respectively; and those of patients who did not receive IFN therapy were 76.6 and 50.6%, respectively. There was a statistically significant difference in overall survival among the three groups ($P = 0.005$) (Fig. 2a). However, there was no statistically significant difference in disease-free survival among the three groups ($P = 0.90$) (Fig. 2b).

Table 2 presents the patterns of cancer recurrence and the treatment details of the recurrences in both groups. Twenty-one (55.3%) of the patients who received peg-IFN therapy after hepatic resection and 17 (44.7%) of the patients who did not receive IFN therapy had HCC recurrences after hepatic resection. Regarding the pattern of recurrence, the proportion of patients who had multiple

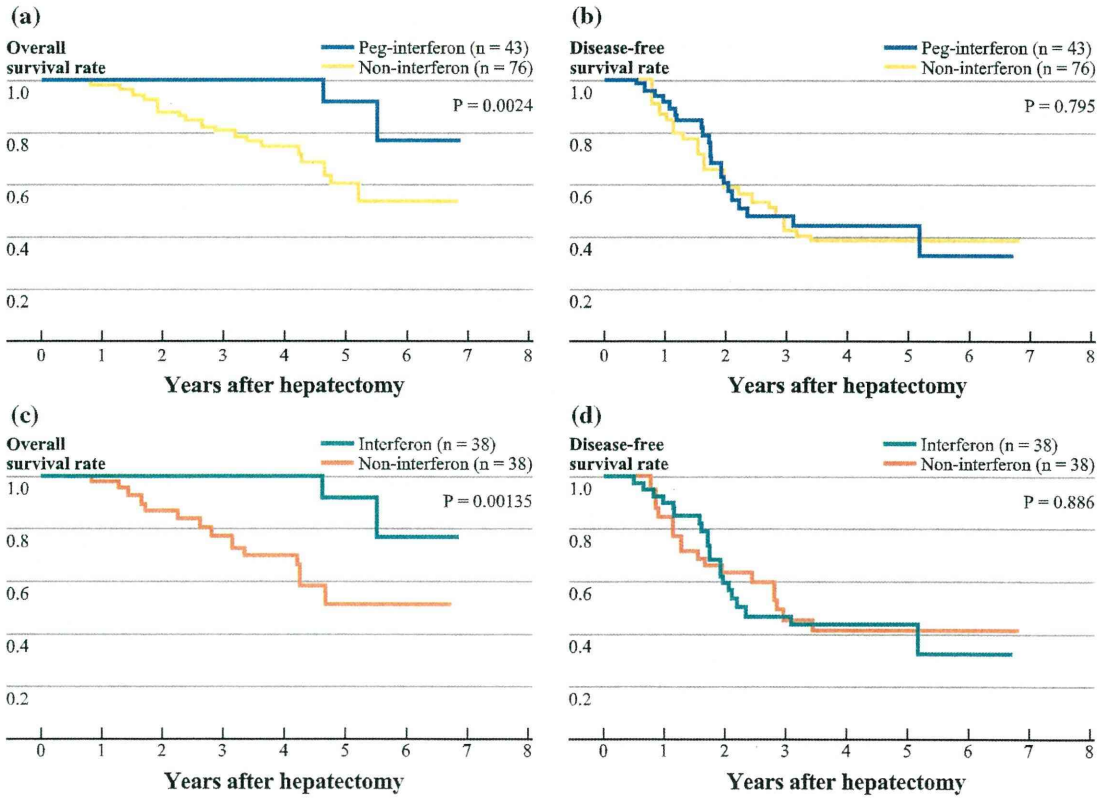

FIG. 1 Overall survival (**a**) and disease-free survival (**b**) of the entire study population of 175 patients with hepatitis C-related HCC with respect to IFN therapy after hepatic resection. Overall survival (**c**) and disease-free (**d**) survival of the matched study population of 76 patients with hepatitis C-related HCC with respect to IFN therapy after hepatic resection

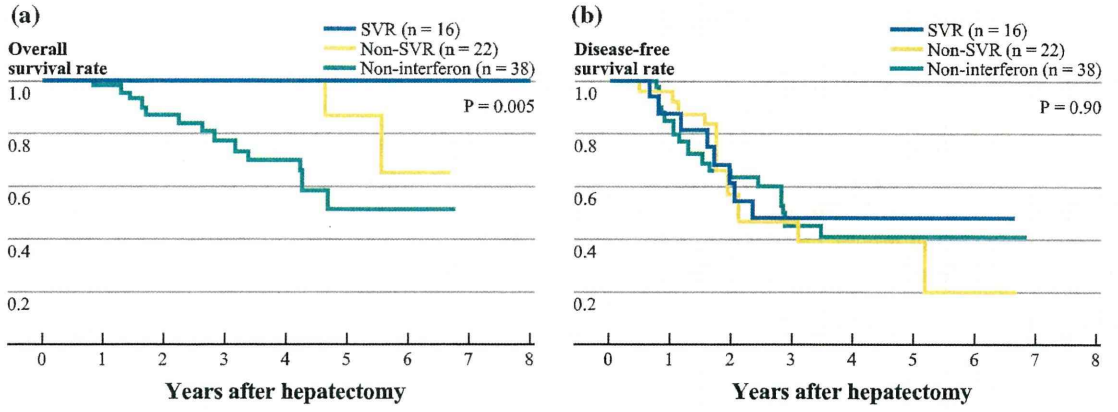

FIG. 2 Overall survival and disease-free survival of patients with hepatitis C-related HCC with respect to SVR after IFN therapy

intrahepatic recurrences (more than four nodules) was significantly lower in the peg-IFN group than in the non-IFN group ($P = 0.0047$). The proportion of patients in whom surgery or RFA was selected for treatment was significantly higher in the peg-IFN group than in the non-IFN group ($P = 0.0346$). Furthermore, regarding re-recurrence of HCC after treatment of the first-recurrent HCC, the 1-year disease-free survival rates of patients after treatment of the first-recurrent HCC was 48.5% in patients ($n = 21$) who received peg-IFN therapy and 12.5% in patients ($n = 17$) who did not receive IFN therapy. There was a statistically significant difference in disease-free survival between the two groups ($P = 0.0012$) (Fig. 3).

A comparison of results of the preoperative liver function test with those of postoperative 1-year liver function tests is presented in Table 3. In patients who received peg-IFN therapy, total bilirubin levels 1 year after surgery were significantly decreased compared with preoperative total bilirubin levels ($P = 0.018$), whereas in patients who did not receive IFN therapy, the total bilirubin level at 1 year after surgery was similar to the total bilirubin level before surgery ($P = 0.107$).

TABLE 2 Recurrence and treatments for recurrence after hepatic resection

	Peg-IFN (+) ($n = 38$)	IFN (−) ($n = 38$)	P value
HCC recurrence[a]: yes	21 (55.3%)	17 (44.7%)	0.359
Pattern of recurrence[b]			0.0047
Intrahepatic (single)	9 (42.9%)	8 (47.1%)	
Intrahepatic (2–3)	10 (47.6%)	1 (5.9%)	
Intrahepatic (multiple)	2 (9.5%)	8 (47.1%)	
Main modalities[b]			0.0346
Repeat hepatectomy	8 (38.1%)	2 (11.8%)	
RFA	8 (38.1%)	4 (23.5%)	
TACE	5 (23.8%)	11 (64.7%)	

peg-IFN pegylated interferon, *RFA* radiofrequency ablation, *TACE* transcatheter arterial chemoembolization

[a] Data expressed as number of patients (percentage of total patients)

[b] Data expressed as number of patients (percentage of patients who had a recurrence)

DISCUSSION

Our results revealed that peg-IFN therapy after hepatic resection improved the outcomes of HCV patients, although the interval of disease-free survival was not prolonged. Peg-IFN therapy after hepatectomy improved hepatic reserve function and suppressed multiple HCC recurrences (more than four nodules). Furthermore, re-recurrence after treatment of first-recurrent HCC after hepatic resection was significantly suppressed in the peg-IFN group compared with that in the non-IFN group. IFN has been reported to exert antitumor effects. IFN increases natural killer cell activity and exhibits antiangiogenic properties.[35,36] IFN has also been reported to be effective in eradicating HCV RNA

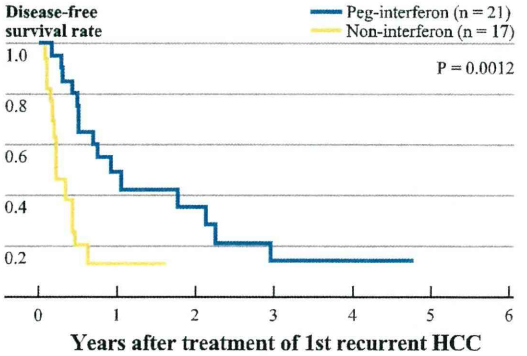

FIG. 3 Comparison of disease-free survival rate after treatment of first-recurrent HCC in patients who received peg-IFN therapy or in those who did not receive IFN therapy

TABLE 3 Comparison of preoperative liver function with 1-year liver function after hepatic resection

	Peg-IFN (+)		*P* value	IFN (−)		*P* value
	Preoperative	1 Year after surgery		Preoperative	1 Year after surgery	
T-Bil (mg/dl)	0.82 ± 0.29	0.71 ± 0.26	0.0189	0.81 ± 0.32	0.92 ± 0.35	0.107
AST (IU/l)	50.1 ± 24.1	45.8 ± 23.5	0.310	42.1 ± 18.9	56.1 ± 26.7	0.0110
ALT (IU/l)	51.3 ± 28.6	36.4 ± 22.8	0.00809	40.3 ± 24.3	49.7 ± 25.8	0.0918
Albumin (g/dl)	3.89 ± 0.80	3.99 ± 0.71	0.251	3.73 ± 0.45	3.75 ± 0.44	0.807

peg-IFN pegylated interferon, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase

from serum and hepatic tissue, thereby preventing deterioration of liver function in patients with HCV infection.[37] IFN prevents worsening of compensated cirrhosis.[18,37] Our results are compatible with those reported in those studies. In the peg-IFN group, most patients with HCC recurrence could undergo curative treatments such as repeat hepatectomy or RFA as a recurrence treatment, because the number of recurrent tumors was usually limited to three. IFN therapy appears to increase survival not only by improving residual liver function and increasing the possibility of radical treatment of recurrences but also by suppressing re-recurrence after the first recurrence of HCC.

The current study also revealed that the overall survival of patients with SVR was significantly better than that of patients without SVR. This result suggests that IFN prolongs the outcomes of patients with HCC after hepatic resection by causing remission of active hepatitis and eradication of HCV RNA in patients who attained SVR after hepatic resection.

In this study, to clarify the impact of peg-IFN therapy on outcomes of HCV-related HCC after hepatic resection, patients who received IFNs such as IFN-α or IFN-β were excluded. RCTs investigating adjuvant effects of IFN after resection or ablation of HCC were performed using IFN-α. Few studies have investigated the effects of peg-IFN plus RBV combination therapy on survival and recurrence after curative resection of HCC. Combination therapy with peg-IFN and RBV has recently been developed, and peg-IFN therapy has resulted in significantly higher SVR rates and better tolerability than treatment with IFN-α.[21,23] In our study, incidence of SVR after hepatic resection was 42.1%, which was higher than that in previous studies that reported an SVR rate of 0–10%.[12–14] The compliance of patients to peg-IFN therapy observed in the present study (68.4%) was higher than that reported elsewhere (approximately 40%).[14] This enhanced efficacy of the peg-IFN formulations might contribute to the prolonged survival of HCC patients after hepatic resection.

In this study, HCC patients who received peg-IFN therapy within 9 months after surgery were enrolled, and HCC patients who experienced recurrence of HCC within 9 months after hepatic resection were excluded from the non-IFN group, because these patients could lose the opportunity to receive IFN therapy for HCC recurrence on being assigned to the peg-IFN therapy group.

Before matching by using the propensity score, the clinical characteristics of the entire study population that can strongly influence outcomes differed significantly between the peg-IFN group and non-IFN group. The proportion of older patients was higher in the non-IFN group than in the peg-IFN group, whereas the proportion of patients who had longer operation times tended to be lower in the non-IFN group than in the peg-IFN group. To overcome bias due to the different distribution of the severity of liver function impairment between the two groups, a one-to-one match was created using propensity score analysis. After matching by propensity score, prognostic variables were appropriately handled, and there was no significant difference in prognostic factors between the two matched groups. This study had a limitation related to the small sample size after propensity score matching. To overcome this, further examination with larger sample sizes is necessary, and the potential efficacy of peg-IFN therapy must be validated in larger prospective RCTs.

CONCLUSIONS

Several previous RCTs investigating the effects of IFN on survival and tumor recurrence after hepatic resection were inconclusive. However, in the current study, peg-IFN therapy following hepatic resection improved the survival rates of hepatectomized patients with HCV-related HCC. The results of this study suggest that peg-IFN therapy is effective as an adjuvant chemopreventive agent after hepatic resection in patients with HCV-related HCC.

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PRECLINICAL STUDY

Mechanistic analysis of the antitumor efficacy of human natural killer cells against breast cancer cells

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Abstract We investigated the role of human natural killer (NK) cells in the peripheral blood (PB) and liver in controlling breast cancer. The proportion of NK cells among liver mononuclear cells was significantly higher than among PB mononuclear cells. Liver NK cells inductively expressed higher levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) than PB NK cells in response to interleukin-2 (IL-2). Liver NK cells displayed higher cytotoxicity against various breast cancer cell lines (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) after IL-2 stimulation than did PB NK cells. Anti-HER2 monoclonal antibody (mAb) promoted the cytotoxicity of both the types of NK cells toward HER2-expressing cell lines. All breast cancer cell lines highly expressed death-inducing TRAIL receptors, death receptor 4, but did not express death-inhibitory receptors (DcR1 and DcR2). Both PB and liver NK cell-induced cytotoxicity was inhibited partially by anti-TRAIL mAb and more profoundly by the combination of anti-TRAIL mAb and concanamycin A, indicating that TRAIL and perforin are involved. IL-2-stimulated liver and PB NK cells exhibited upregulated expression of CXCR3, which bind to the chemokines CXCL9, CXCL10, and CXCL11 secreted by breast cancer cells. We also found that IFN-γ promoted the production of CXCL10 from breast cancer cells. The results of this study show that IFN-γ secreted from NK cells likely promotes the production of CXCL10 from breast cancer cells, which in turn accelerates the migration of CXCR3-expressing NK cells into the tumor site. These findings suggest the possibility of a therapeutic approach by either activation of endogenous PB and liver NK cells or adoptive transfer of in vitro-activated autologous NK cells.

Keywords NK cells · TNF-related apoptosis-inducing ligand (TRAIL) · TRAIL-receptors · ADCC · Chemokine · Breast cancer

Abbreviations

NK	Natural killer
TRAIL	TNF-related apoptosis-inducing ligand
PBMC	Peripheral blood mononuclear cell
LMNC	Liver mononuclear cell
mAbs	Monoclonal antibodies

Introduction

Natural killer (NK) cells, the frontline defense in cellular immunity, exert an effector function on neoplastic cells, modified cells, and invading infectious microbes without the necessity for priming [1, 2]. Although, NK cells might

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play an important role in prevention of both early and metastatic cancer, the role of NK cell activity in controlling breast cancer is still controversial and few studies have addressed whether enhancing this activity is of clinical benefit to breast cancer patients.

A variety of mechanisms are involved in controlling neoplastic cells by NK cells, one of which is the direct release of cytolytic granules that contain perforin, granzymes, and granulysin by exocytosis to kill target cells (i.e., the granule exocytosis pathway) [3, 4]. Most mature human NK cells in peripheral blood (PB) constitutively express granzyme B and perforin, and have basal cytotoxicity against NK-sensitive targets. Cytokine exposure with interleukin (IL)-2 or IL-15 is known to increase the baseline granzyme B and perforin abundance and cytotoxic activity of NK cells, and also converts basal NK cytotoxicity to lymphokine-activated killing. Another mechanism is mediated by death-inducing ligands such as Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Fas, a TNF family protein, is expressed on breast cancer cell membranes [5, 6], suggesting that activation of the Fas/FasL pathway induces apoptosis mediated by caspase activation. An additional mechanism is involved when HER2-overexpressing breast cancer cells are targeted because differential levels of HER2 expression in normal versus HER2-overexpressing tumor cells, together with the clear involvement of HER2 in tumor progression, make HER2 an ideal target for therapeutic approaches. NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) is thought to contribute to the therapeutic effects of monoclonal antibodies (mAbs) specifically directed against the extracellular domain of HER2 (trastuzumab).

NK cells are abundant in the liver in contrast to their relatively small percentage in the peripheral lymphatics and other lymphatic organs in rodents [7–9] and humans [10]; however, the underlying reason for the anatomically biased distribution of NK cells has not been elucidated. In addition, liver NK cells have been shown to mediate higher cytotoxic activity against tumor cells than PB NK cells do in rodents [7–9, 11]. However, these functional differences between PB and liver NK cells have not been extensively investigated in humans because of the limited availability of appropriate samples. In this study, we extracted NK cells from allograft liver perfusates in clinical liver transplantation and examined the quantitative and qualitative cytotoxic functions of those liver NK cells targeting various breast cancer cell lines in comparison with PB NK cells. Through the experiments, we attempted to define whether PB NK cells can recognize and kill breast cancer cells, and whether liver resident NK cells can hinder metastasis of breast cancer to the liver, to assess the potential therapeutic use of NK cells, i.e., by either activation of endogenous NK cells or adoptive transfer of in vitro-activated autologous NK cells. As the therapeutic efficacy of endogenous or exogenous NK cells likely depends on their migration and accumulation at tumor metastasis sites, we further analyzed the expression of receptors and ligands for chemokines secreted from breast cancer cells on PB and liver NK cells.

Materials and methods

Isolation of liver and PB lymphocytes

Liver mononuclear cells (LMNCs) were obtained from liver perfusates in clinical living donor liver transplantation as previously described [10]. LMNCs were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). PB mononuclear cells (PBMCs) were also isolated by gradient centrifugation with Separate-L from heparinized PB from healthy volunteers and liver transplant donors. LMNCs and PBMCs were suspended in DMEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sanko Chemical Co., Tokyo, Japan), 25 mmol/l HEPES buffer (Gibco), 50 μmol/l 2-mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco) (10% DMEM). The ethics committee at Hiroshima University Hospital approved this study.

Cell culture

LMNCs and PBMCs were cultured with human recombinant IL-2 (100 Japanese reference U/ml; Takeda, Tokyo, Japan) in DMEM at 37°C in a 5% CO_2 incubator. Cells were harvested for further analyses after 5 days in culture. Cell viability was assessed by the dye-exclusion test.

Isolation of NK cells

LMNCs and PBMCs were separated into a $CD3^{-}CD56^{+}$ NK cell fraction and a non-NK cell fraction (T cells, NKT cells, B cells, and monocytes/macrophages) by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), using the human NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated fractions was assessed by FCM, and only preparations with purities >90% were used for functional studies.

Cell lines

The human breast cancer cell lines were obtained as follows: MDA-MB-231 and MDA-MB-468 were from ATCC