Table 3. Characteristics of patients who received autologons stem cell transplantation (SCT)

Case no.	Sex	Age at diagnosis (months)	Laterality	Metastasis at diagnosis	Treatment	Metastasis after treatment	Treatment after metastasis	Chemotherapy after metastasis
1	F	3	Bilateral	None	Rx (50.7 Gy) + enucleation (lt)	Brain, spinal cord	Spine, 40 Gy Brain, 40 Gy	VCR + CY + ADR; CY + CDDP
2	M	10	Bilateral	None	Rx (49.4 Gy)+ enucleation (lt)	Brain	Brain, 39.6 Gy; spine, 21 Gy	VCR + CY + ADR; CDDP + VP-16
3	M	13	Unilateral	None	Rx (50 Gy)	Brain	Brain, 50 Gy + enucleation	CDDP + VP-16; CDDP + VP-16 + ADR
4	F	41	Unilateral	None	Rx (46 Gy) + HIT	Bone, BM	Bone, 40 Gy	VCR + CY + ADR; CDDP + VP-16
5	F	16	Unilateral	BM	Rx (41 Gy) + enucleation + Cx	Bone	–	VCR + CY + ADR; CDDP + VP-16
6	F	18	Unilateral	None	Rx (46 Gy) + HIT + PC + CTT + IVI + enucleation	Orbit, BM	–	VCR + CY + ADR + CDDP; CBDCA + VP-16

ANC, absolute neutrophil count; Plt, platelets; Rx, radiotherapy; lt, left; rt, right; Ret, reticulocytes; HIT, heat-inducing thermotherapy; Cx, chemotherapy; PC, photocoagulation; CTT, chemothermotherapy; IVI, intravitreal injection; BM, bone marrow; CFU, colony-forming unit; PB, peripheral blood stem cell; VCR, vincristine; CY, cyclophosphamide; ADR, doxorubicin; CDDP, cisplatin; VP-16, etoposide; CBDCA, carboplatin; L-PAM, melphalan; TEPA, thiotepa; CNS, central nervous system; LN, lymph node; NED, no evidence of disease; DOD, dead of disease; NE, not evaluable

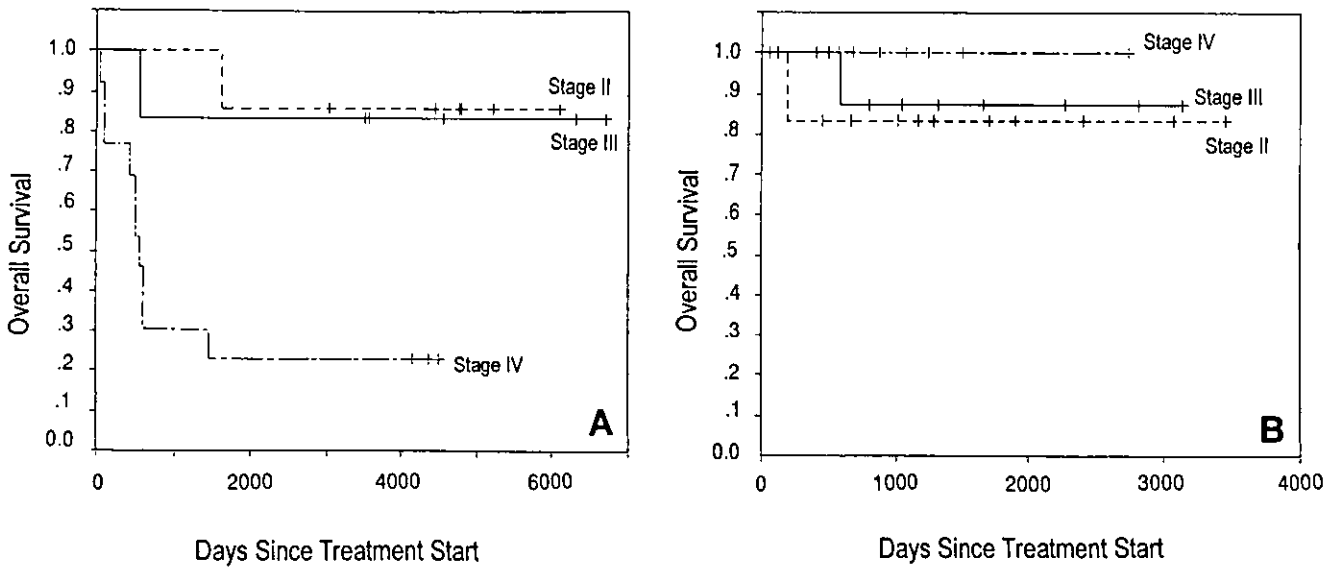

Fig. 2A,B. Overall survival according to the St. Jude staging system. **A** Patients treated before 1990; **B** patients treated after 1990

preferable to alternating regimens A and B (etoposide and cisplatin) for patients with less advanced disease, considering the risk of a second malignancy and the risk of renal toxicity with the drugs in regimen B.

High-dose chemotherapy (HDC) with autologous stem cell transplantation (SCT) for advanced retinoblastoma

Although adjuvant chemotherapy contributes to the prevention of recurrence in some patients with advanced or potentially advanced retinoblastoma, as described above, the prognosis of patients with metastatic retinoblastoma remains poor. In particular, patients with CNS disease seldom achieve longterm survival. Because retinoblastoma is one of the highly chemosensitive tumors, a treatment strategy including dose-escalation of chemotherapeutic agents, using stem cell support, is rational. A limited number of studies and case reports have suggested that HDC with autologous stem cell rescue may be beneficial for patients with metastatic retinoblastoma.[23–31]

Namouni et al.[25] conducted a study of HDC that consisted of carboplatin, etoposide, and cyclophosphamide (CARBOPEC), followed by autologous SCT in 25 patients.

Timing of SCT after relapse (months)	Conditioning (mg/m^2)	Stem cell source (/kg)	ANC (>500/μl)	Plt (>50 000/μl)	Ret (>1%)	Result	Metastasis after SCT	Sequelae
5	CDDP, 90 CY, 120 L-PAM, 180	BM 1.07×10^8	Day 18	Day 67	Day 42	DOD	Th12-L1, CNS (24 months)	NE
5	CDDP, 90 CY, 120 L-PAM, 180	BM 1.01×10^8	Day 26	Day 32	Day 21	DOD	Rt neck LN (4 months)	NE
6	CDDP, 90 CY, 120 VP-16, 800	BM 1.3×10^8	Day 12	Day 12	Day 23	NED (148+ months)	None	Cataract, epilepsy, brain necrosis
7	L-PAM, 180 VP-16, 800 CBDCA, 1600	BM 1.2×10^5 (CFU)	Day 10	Day 11	Day 16	NED	None (87+ months)	None
6	L-PAM, 180 VP-16, 800 CBDCA, 1600	BM 1.65×10^8	Day 14	Day 51	Day 46	NED (81+ months)	None	None
7	L-PAM, 160 CY, 120 TEPA, 500	PB 4.7×10^6	Day 11	Day 16	Day 14	NED (12+ months)	None	None

Although the 3-year disease-free survival was 67.1%, no patients with CNS disease survived. Dunkel et al.[28] reported 4 patients with orbit and bone marrow metastatic retinoblastoma, who underwent HDC consisting of carboplatin and thiotepa, with or without etoposide. All 4 patients had an event-free survival of 46–80 months after the diagnosis of metastatic disease. Kremens et al.[30] treated 4 patients with HDC consisting of thiotepa, etoposide, and carboplatin, and they treated 1 patient with carmustine (BCNU), cyclophosphamide, and etoposide. One patient developed a meningeal relapse 10 months after the HDC, but this was successfully treated by partial resection and conventional chemotherapy, and resulted in long-term survival, for 105 months after completion of the treatments.

We treated six patients with metastatic retinoblastoma using induction multi-agent chemotherapy followed by HDC and autologous SCT (Matsubara H et al. High dose chemotherapy for retinoblastoma. Unpublished work). In five patients, 40–50 Gy of radiation was administered to the metastatic lesion before the HDC was begun. Table 3 summarizes the patient characteristics. Five patients received melphalan-based HDC and the remaining patient received a combination of cyclophosphamide, cisplatin, and etoposide. Four of the six patients had event-free survivals that ranged from 12 to 148 months. All patients without CNS involvement survived disease-free after the HDC. One patient with CNS metastatic retinoblastoma (case 3) continues to be disease-free at 148 months after a treatment combination of HDC and cranial radiation, although the patient is compromised with a series of sequelae including brain necrosis, epilepsy, and cataract.

It seems that HDC with autologous SCT is effective in patients with metastatic retinoblastoma, although variations in the HDC regimens made it difficult to judge the objective safety and efficacy of autologous SCT. Considering the fact that HDC only works as an intensive consolidation chemotherapy, it is as important to establish the most effective multidisciplinary treatment sequence as it is to find good HDC regimens. Especially for patients with CNS involvement, a safer and more effective modality is necessary to prevent late sequelae secondary to the additive toxicity induced by HDC and cranial radiation.

Future directions

Because of the small number of patients with retinoblastoma and the diversity of the disease characteristics in individual patients, there have been no clinical trials to determine whether to recommend a particular regimen, or to identify specific criteria in patients who would benefit from chemotherapy. In this regard, it is important to develop a large cooperative study group which could accumulate a sufficient number of patients, as well as a supportive statistical and data-management center to ensure the quality of the study design and the data. Such an infrastructure would allow us to conduct well-designed prospective controlled trials, which are warranted to establish a standard treatment strategy for patients with extraocular retinoblastoma.

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Review Article

Reduced-intensity Hematopoietic Stem Cell Transplantation (RIST) for Solid Malignancies

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Review Article

Reduced-intensity Hematopoietic Stem Cell Transplantation (RIST) for Solid Malignancies

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Reduced intensity stem cell transplantation (RIST) is a new approach of stem cell transplantation, which has shown promising features as reported in multiple phase I and II studies. Elderly patients, who are not eligible for conventional myeloablative hematopoietic stem cell transplantation (HSCT), are now treatable with RIST. It has also reduced regimen-related toxicity and provided better prognosis in short-term follow-up than conventional HSCT. Among solid tumors, metastatic renal cell carcinoma was found to respond well to RIST. Clinical studies are currently being conducted to evaluate the efficacy of RIST in other types of solid tumors. However, the mechanism of graft-versus-host disease (GVHD) and graft-versus-tumor (GVT) effects remains unclear. More knowledge on the mechanism is crucial to enhance the antitumor effect and to improve the prognosis further.

Key words: graft-versus-tumor effects – graft-versus-host disease – renal cell carcinoma – allogeneic hematopoietic stem cell transplantation – breast cancer

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION AS AN IMMUNE THERAPY

Allogeneic hematopoietic stem cell transplantation (allo-SCT) for the treatment of hematological malignancies was originally based upon the effect of a myeloablative preparative regimen. A preparative regimen using high-dose chemoradiotherapy would suppress the host's immune response and eradicate the residual tumor cells. Marrow was infused to restore hematopoiesis (1). In combination with preceding induction and consolidation cytotoxic chemotherapy, myeloablative preparative regimens followed by allo-SCT were supposed to eradicate the residual underlying diseases.

However, it was found that allogeneic cells were responsible for immunological responses against tumor cells. This is called a graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) effect (2). Evidence supporting this hypothesis includes (i) lower incidences of relapse in patients receiving allo-SCT than in those receiving autologous SCT (3); (ii) higher risk of relapse in patients receiving syngeneic SCT (4); and (iii) lower risk of relapse in patients with acute and/or chronic graft-versus-host disease (GVHD) than those without these conditions (5). Furthermore, GVL or GVT effects were found to be mediated by lymphocytes, especially T cells, based on the clinical findings of (i) higher risk of relapse after T-cell depletion than non-depleted SCT (6); and (ii) therapeutic effects of donor lymphocyte infusion (DLI) (7). In particular, chronic myeloid leukemia (CML) responds well to DLI, and most patients with CML who relapse following allo-SCT can achieve remission with DLI (8). Based on these findings, allo-SCT is now regarded as one of the available immune therapies.

REDUCED-INTENSITY STEM CELL TRANSPLANTATION (RIST)

The high-dose chemotherapy and radiation used as preparative regimen for allo-SCT are associated with a considerable morbidity and mortality (9). This approach has therefore been restricted to young patients without co-morbidities. The majority of patients with hematological malignancies are ineligible for high-dose chemotherapy or radiotherapy because of their old age and co-morbidities. Although allo-SCT is the most powerful treatment for refractory hematological malignancies, only a small proportion of these patients have the opportunity to undergo this treatment.

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Recently, a new strategy for transplantation using a reduced-intensity or non-myeloablative preparative regimen has been developed to reduce regimen-related toxicity (RRT) while preserving adequate antitumor effects (10–14). Various regimens with different intensity can be categorized roughly into two intensity groups: (i) reduced-intensity regimens which retain a certain degree of RRT and require hospitalization; and (ii) minimally myelosuppressive regimens which rely on post-grafting immunosuppression to permit engraftment (15,16). The aim of post-grafting immunosuppression is to control GVHD and to suppress residual host-versus-graft (HVG) effects that would impede engraftment.

These reduced-toxicity regimens are frequently termed 'non-myeloablative' and 'reduced-intensity' regimens. At present, a variety of preparative regimens have been developed. Both myelosuppression and immunosuppression vary widely among them. According to a working definition, a truly non-myeloablative regimen should allow prompt hematopoietic recovery (within 28 days of transplantation) without stem cell rescue, and mixed chimerism usually occurs upon engraftment (15,16). These regimens do not ablate host immunity and depend on the activity of donor T cells to achieve engraftment. The regimen of 2 Gy total body irradiation (TBI) with or without fludarabine reported by the Seattle Transplantation Team (12) is classified as a truly non-myeloablative regimen. In contrast, autologous hematopoietic recovery does not occur without stem cell support after the other regimens such as fludarabine/busulfan and fludarabine/cyclophosphamide, and they are termed reduced-intensity preparative regimens.

PRECLINICAL MODEL OF NON-MYELOABLATIVE SCT

The Seattle Transplantation Team reported the results of preclinical canine studies on non-myeloablative SCT. The researchers considered that two immunological barriers must be overcome in the setting of allo-SCT (17). One is the GVHD, and the other is the rejection or HVG reaction. Both reactions are mediated by T lymphocytes, suggesting that immunosuppressive agents given after allo-SCT to control GVHD might modulate HVG reactions. The latter feature would allow minimization of the high-dose chemotherapy given before allo-SCT for host suppression.

Animal models demonstrated a dose–response relationship between TBI and engraftment (18). In random-bred dogs, a single fraction of 920 cGy TBI, corresponding to 1500 cGy fractionated TBI, resulted in engraftment of dog leukocyte antigen (DLA)-identical littermate marrow in all cases. When the dose was decreased by 50%, the majority of dogs rejected their grafts. At the reduced dose, the addition of post-grafting prednisone did not enhance engraftment, while cyclosporin given for 5 weeks led to engraftment in all of the animals. When the TBI dose was decreased further to 200 cGy, cyclosporin only allowed engraftment for 3–4 months, after which the grafts were rejected. The combination of methotrexate and cyclosporin resulted in engraftment in two out of five animals, but the rest rejected. A combination of mycophenolate mofetil (MMF) and cyclosporin given for 4 and 5 weeks after transplantation was evaluated for its effect on engraftment. The regimen was capable of both controlling GVHD and preventing graft rejection by suppressing a GVH reaction, with 11 of 12 dogs demonstrating stable engraftment of marrow from DLA-identical littermates (19).

They further investigated whether the major role of TBI is to create marrow space or to provide host immunosuppression (20). They irradiated the central lymph node chain from the neck to the upper abdomen with 450 cGy before allo-SCT, and administered MMF and cyclosporin after allo-SCT. At 6 weeks post-transplant, donor cells were present in non-irradiated marrow spaces, suggesting that radiation was not essential to create marrow space for engraftment. After 1 year, DLI was given to the animals and recipient cells disappeared within 9 weeks. These findings indicate that engraftment might be accomplished by blocking HVG reactions and inducing the GVH reaction, and that high-dose cytotoxic chemotherapy and radiotherapy could be eliminated from the preparative regimens.

RATIONALE OF ALLO-SCT FOR SOLID TUMORS

Several findings justify allo-SCT for solid tumors: (i) GVT effects can target tissue-specific polymorphic antigens which are not derived from hematopoietic lineages; (ii) some solid tumors are sensitive to immunotherapy, such as renal cell carcinoma (RCC), melanoma and ovarian cancer; (iii) antigens restricted to the tumor could stimulate tumor-specific allo-immunity in contrast to defective T cells in the tumor-bearing host; and (iv) in theory, all carcinomas arising from epithelial tissues such as keratinocytes, fibroblasts, exocrine glands, hepatobiliary trees and the gastrointestinal tract, which are targets of acute and chronic GVHD, should be susceptible to a GVT effect.

Before clinical trials were initiated, murine models have provided some evidence for a GVT effect (21,22). Among animals inoculated with mammary adenocarcinoma cells, the recipients of allo-SCT showed better survival than did those of syngeneic SCT (21). Further studies provided evidence that murine mammary adenocarcinoma cells expressed minor histocompatibility antigens (mHas) that could be targeted by alloreactive donor T cells in the setting of allogeneic but not autologous bone marrow transplantation (23). Prigozhina et al. demonstrated in animal models that effective eradication of tumor cells as well as leukemic cells can be achieved following allo-SCT using non-myeloablative preparative regimens (24).

The earliest clinical evidence supporting the existence of a GVT effect in a solid tumor was observed in a patient with metastatic breast carcinoma undergoing fully myeloablative SCT for relapsed acute myeloid leukemia. The incidental regression of a metastatic lesion of breast carcinoma raised the possibility of a responsible GVT effect (25). Regression of liver

metastasis in association with severe acute GVHD was reported in a woman transplanted for metastatic breast carcinoma. The researchers demonstrated that allogeneic T cells collected during GVHD and cultivated were able to mediate a cytotoxic effect against breast cancer cell lines (26), suggesting that disease regression resulted from donor T cells targeting broadly expressed mHas. Since then, similar anecdotal reports have been published concerning a possible GVT effect in lung cancer (27), ovarian cancer (28), colon cancer (29), neuroblastoma (30), pancreas cancer (31,32) and ependymoma (33). Porter et al. conducted a phase I clinical trial to determine whether a GVT effect could be observed after primary DLI without stem cell support in patients with primary cancers (34). Three of four patients with acute GVHD and late chimerism responded to primary DLI. These findings indicate that the GVT effect does occur in the setting of allo-SCT for solid tumors.

CLINICAL TRIALS FOR SOLID TUMORS

Metastatic Renal Cell Cancer (RCC)

In 1997, Childs et al. initiated a clinical trial to evaluate GVT effects in metastatic RCC (35). Chemotherapy is ineffective in the majority of cases and does not prolong survival. However, RCC has a distinct nature from that of other solid tumors. There is increasing evidence that they may be susceptible to T-cell immune responses. Biopsy of spontaneously regressing lesions has shown tumor-infiltrating lymphocytes with predominant $CD8^+$ T cells exhibiting major histocompatibilty complex (MHC) class I restricted cytotoxicity against autologous tumor targets (36). Furthermore, unlike most solid tumors, RCC is susceptible to cytokines such as interleukin-2 (IL-2) and interferon-α (37), suggesting that T cells represent the principle effector.

Childs' group treated 19 patients with metastatic RCC (35). The preparative regimen consisted of fludarabine 25 mg/m^2 for 5 days and cyclophosphamide 60 mg/kg for 2 days. Cyclosporin, used to prevent GVHD, was withdrawn early in patients with mixed T-cell chimerism and/or disease progression. Patients without response received up to three courses of DLI. At the time of the last follow-up, nine of the 19 patients were alive 287–831 days after transplantation (median follow-up, 402 days). Two died of transplantation-related causes, and eight from progressive disease. In 10 patients, metastatic disease regressed: three had a complete response, and seven had a partial response. The patients who had a complete response remained in remission 27, 25 and 16 months after transplantation. Results of this clinical trial were updated in 2002 (38). Clinical response is significantly associated with the development of GVHD. There is a 4–6 month interval between transplantation and development of a GVT effect, and patients with rapidly progressive diseases are unlikely to benefit from RIST. Disease response was observed most commonly in patients with pulmonary metastases of clear-cell histology without other organ involvement. Some patients who had failed to respond to interferon-α prior to transplantation achieved responses following administration of a low dose of this agent after transplantation.

After the first report on RIST for RCC, several phase I/II studies have been reported (Table 1) (39–44). Response rates varied widely from 0 to 57%, but it should be noted that some responses were reported in seven of the nine studies. While long-term prognosis remains unknown, response to allo-SCT has been confirmed in some independent studies. Rini et al. described regression of primary kidney tumors, a rare event among responders to cytokine-based therapy (39). According to a European retrospective survey (45), allo-SCT was used in <20 cases of solid tumors until 1997; since then it increased to 159 in 2002, mainly for RCC.

We also initiated a phase I clinical trial on RIST for metastatic RCC (46). From June 2000 to April 2002, nine patients received peripheral blood stem cell transplantation from a

Table 1. Clinical trials on RIST for metastatic renal cell carcinoma

Reference	Donor	No. of patients	Preparative regimen	GVHD prophylaxis	Response rates
Childs et al. (35)	An HLA-identical or one locus-mismatched related donor	19	CY/Flu	CSP	53%
Childs and Barrett (38)	HLA-identical and one locus-mismatched related	52	CY/Flu	CSP	48%
Rini et al. (39)	An HLA-identical sibling	12	CY/Flu	Tacrolimus and MMF	33%
Bregni et al. (40)	An HLA-identical sibling	7	CY/Flu	CSP and MTX	57%
Blaise et al. (42)	An HLA-identical sibling	25	ATG/BU/Flu	CSP	4%
Ueno et al. (43)	An HLA-identical related or matched unrelated donor	15	Melphalan/Flu	Tacrolimus and MTX	27%
Pedrazzoli et al. (41)	An HLA-identical sibling	7	CY/Flu	CSP and MTX	0%
Hentschke et al. (44)	An HLA-identical related or matched unrelated donor	10	2 Gy TBI/Flu*	CSP and MMF	0%
Nakagawa et al. (46)	An HLA-identical sibling	9	ATG/BU/Flu	CSP	11%

CY, cyclophosphamide; Flu, fludarabine; CSP, cyclosporin; MMF, mycophenolate mofetil; MTX, methotrexate; ATG, anti-thymocyte globulin; BU, busulfan; TBI, total body irradiation.
*Recipients receiving transplants from unrelated donors were given thymoglobulin.

human leukocyte antigen (HLA)-identical sibling donor. The conditioning regimen consisted of fludarabine 180 mg/m^2 or cladribine 0.66 mg/kg, plus busulfan 8 mg/kg and rabbit anti-thymocyte globulin (ATG). GVHD prophylaxis consisted of cyclosporin 3 mg/kg alone. All of the patients achieved engraftment, with no grade III–IV non-hematological RRT, and complete donor cell type chimerism was achieved without additional DLI by day 60. Acute and chronic GVHD was seen in four patients each. One patient achieved partial remission (response rate 11%) and, as of July 2003, six patients are alive with a median follow-up of 22.5 months. The actuarial overall survival rate was 74% at 2 years. We followed all the 26 patients who were referred to our institute for RIST and were subject to HLA typing. Transplanted patients (n = 9) showed significantly higher overall survival rate than those who had not received RIST (n = 17) (Fig. 1A, P = 0.016). We compared the overall survival rates between 12 patients with matched donors and the other 14 patients without them (Fig. 1B). The 1-year actuarial survival rates were 74 and 48% in patients with donors and those without them, respectively (P = 0.088). This study confirmed the feasibility of allo-SCT for metastatic RCC, and suggests that it might improve prognosis of patients with metastatic RCC. Further phase II or III studies are warranted.

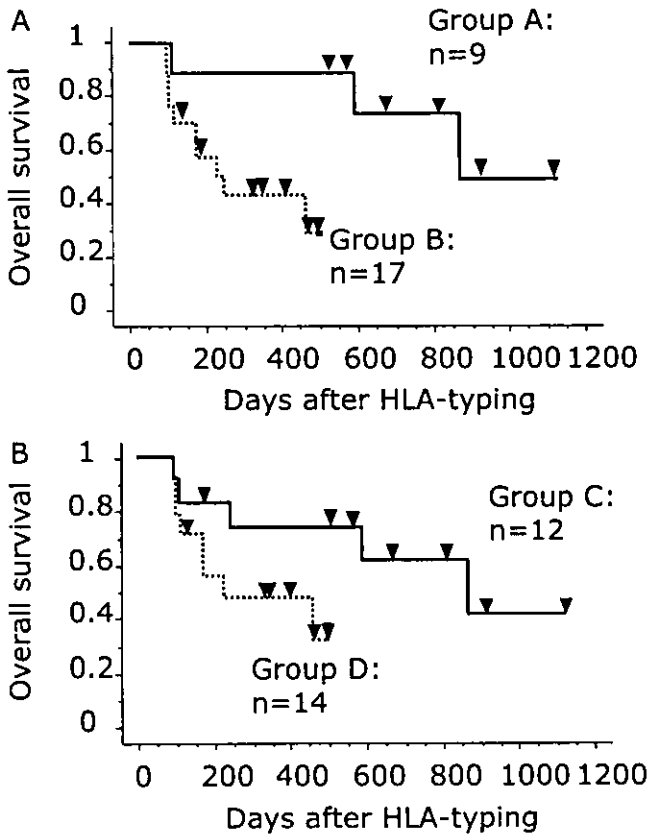

Figure 1. Kaplan–Meier estimates of the overall survival rates following HLA typing. (A) A comparison of overall survival rates between transplanted and non-transplanted patients. The overall survival rate was significantly higher in transplanted patients than in non-transplanted patients (P = 0.016). (B) A comparison between patients with an HLA-matched donor and those without. A trend toward a better survival was observed in patients with an HLA-matched donor (P = 0.088). Group A, transplanted patients (n = 9); group B, patients who had not received transplantation (n = 17); group C, patients with an HLA-matched donor (n = 12), including nine transplanted patients; group D, patients without an HLA-matched donor (n = 14).

Breast Cancer

After the first case report by Eibl et al. (26), Ueno et al. reported the results of a feasibility study on conventional myeloablative allo-SCT for metastatic breast cancer in 16 patients (47,48). This study included patients without progressive disease. The preparative regimen consisted of cyclophosphamide, carmustine and thiotepa. GVHD prophylaxis was mainly tacrolimus and methotrexate. The responses were complete response (n = 1), partial response (n = 5) and stable disease (n = 8) in the 15 evaluable patients. Two patients responded during acute GVHD following the withdrawal of immunosuppression.

Ueno et al. further investigated the feasibility of RIST for metastatic breast cancer (43). A total of eight patients received allo-SCT following fludarabine and melphalan. Three patients showed some clinical responses (complete response two, minor response one). Metastatic lesions resolved 3 months after development of chronic GVHD in one patient, and the other two patients demonstrated tumor response at 13 and 17 months after transplantation. The delayed response was comparable with that in RIST for RCC. Since fludarabine and melphalan produce little cytoreduction in metastatic breast cancer and the underlying disease progressed immediately after transplantation in more than half of the patients, it is reasonable to assume that the disease response was attributable to a GVT effect.

Since their reports, GVT effects against breast cancer have been confirmed by other researchers (40–42,49) (Table 2).

Melanoma

Childs and Srinivasan treated 11 patients with metastatic melanoma (50). This study highlights some of the potential problems in applying RIST for some solid tumors. Death from rapid disease progression occurred before day 100 in five patients. Although three patients achieved partial regression, their responses occurred early in the courses of RIST with a short duration, suggesting that these responses were attributable to chemotherapy effects related to preparative regimens rather than GVT effects. One patient had delayed regression of several subcutaneous metastatic nodules. The investigators speculated that RIST should be limited to a minority of melanoma patients who have slow-growing diseases.

Other Cancers

There is little information on the efficacy of allo-SCT for most solid tumors. Some anecdotal reports have been published on allo-SCT for a variety of cancers (28,31,44,51–54). A case report and a small case series of RIST for metastatic ovarian

Table 2. Experience on allo-SCT for metastatic breast cancer

Reference	Donor	No. of patients	Preparative regimen	GVHD prophylaxis	Response rates*
Ueno et al. (48)	An HLA-identical sibling	16	CBT	Tacrolimus and MTX+	40%
Ueno et al. (43)	An HLA-identical related or matched unrelated donor	8	Melphalan/Flu	Tacrolimus and MTX	25%
Bregni et al. (40)	An HLA-identical sibling	6	CY/Flu	CSP and MTX	33%
Blaise et al. (42)	An HLA-identical sibling	17	ATG/BU/Flu	CSP	12%
Pedrazzoli et al. (41)	An HLA-identical sibling	2	CY/Flu	CSP and MTX	100%
Hentschke et al. (44)	An HLA-identical related or matched unrelated donor	1	2 Gy TBI/Flu	CSP and MMF	0%

CY, cyclophosphamide; Flu, fludarabine; CSP, cyclosporin; MMF, mycophenolate mofetil; MTX, methotrexate; ATG, anti-thymocyte globulin; BU, busulfan; TBI, total body irradiation; CBT, cyclophosphamide, carmustine, thiotepa.
*Response includes complete and partial responses.
+Two patients received cyclosporin and methylprednisolone.

cancer and colorectal cancer have been published recently (28,42,44,53,54). These tumors may be promising candidates for allo-SCT; however, it should be noted that both ovarian and colorectal cancer are susceptible to chemotherapy, making it difficult to conclude that disease regression was attributable to a GVT effect.

We evaluated a total of 14 patients with refractory non-renal solid tumors (four rhabdomyosarcoma, two melanoma, two neuroblastoma, two cholangiocarcinoma, two other sarcomas and two other carcinomas) who underwent RIST according to our institutional phase I protocol (52,55). The conditioning regimen and GVHD prophylaxis were the same as those for metastatic RCC. All patients but one with melanoma achieved complete donor chimerism without DLI. Only three patients showed grade II–IV acute GVHD and two showed chronic GVHD. Four patients died before day 100 after RIST and another four after day 100. Seven out of the eight patients died of disease progression. Although comprehensive evaluation of the GVT effect is impossible due to the diversity of the diseases, it is remarkable that there are two patients with disease-free survival longer than 11 months after RIST. One is a 7-year-old female with metastatic neuroblastoma which recurred after autologous bone marrow transplantation. The other is a 16-year-old female with metastatic alveolar type rhabdomyosarcoma. Both were transplanted when they had a small volume of residual disease compared with other patients with sarcoma. Among patients with carcinomas, a 56-year-old male with cholangiocarcinoma showed objective tumor regression which did not satisfy the criteria for partial regression (Fig. 2). There was no apparent correlation between GVHD and a GVT effect.

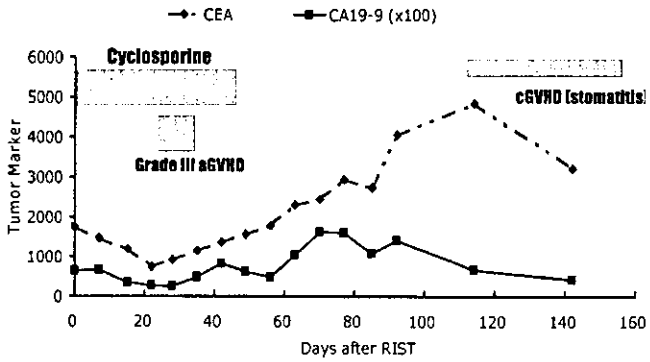

Figure 2. Clinical course of a patient with metastatic cholangiocellular carcinoma. A 56-year-old male with cholangiocarcinoma showed objective tumor shrinkage but not sufficiently satisfactory to regard it as partial remission.

MECHANISM

The precise mechanism of the GVT effect remains unknown. The lack of information on tumor target antigens and immune mediators for GVT effects does not allow us to predict which diseases will respond to RIST.

Disease regression associated with cyclosporin withdrawal, complete donor chimerism and GVHD provides evidence that cytotoxic donor T cells play an important role in this response. RCC cells express a broad range of mHas that could render them susceptible to a GVT effect (56). These findings suggest that both broadly expressed mHas and antigens restricted to RCC cells may be a target of a GVT effect. Recent studies have demonstrated that distinct T-cell populations recognizing tumor-specific antigens and/or mHas are involved in the GVT effect (57). T-cell clones attacking both RCC cells and hematopoietic cells of the recipients were isolated from responding patients (58). Retrospective clinical studies and *in vitro* studies using clinical samples demonstrated that cytotoxic T cells against leukemia-specific antigens or hematopoiesis-restricted mHas can induce remission in allo-SCT for acute leukemia (59–61). In animal models, adoptive transfer of HA-1- and HA-2-specific cytotoxic T lymphocytes generated *in vitro* can be used as immunotherapy to treat hematological malignancies relapsing after allo-SCT (62,63). Using these cytotoxic T cells, GVT effects can be separated from GVHD (64). In contrast to allo-SCT for hematological malignancies, little information is available concerning target

antigens and cytotoxic T cells in allo-SCT for solid malignancies, and further studies are warranted.

Some investigators suggested that innate immunity plays an important role in the development of a GVT effect. Natural killer (NK) cells have been studied intensively, since they are capable of mediating a GVL effect in acute myeloid leukemia without causing GVHD (65). Igarashi et al. reported that allogeneic NK cells with killer immunoglobulin-like receptor ligand incompatibility play an important role in cytotoxicity against melanoma and renal cell carcinoma cells (66). Furthermore, Teshima et al. reported that the local cytokine storm associated with the early phase of allogeneic transplantation plays an important role in GVHD (67). The tumor progression and regression in concordance with corticosteroid use and discontinuation observed in our study (46,68) are compatible with their suggestion, since the cytokine production is readily suppressed by corticosteroid.

Stelljes et al. recently reported an interesting animal study using allogeneic parent-into-F(1) murine transplantation models [BALB/c or C57BL/6→[C57BL/6 × BALB/c]F(1)] with different tumors derived from either parental strain (69). They provided experimental proof of a donor $CD8^+$ T cell-mediated tumor-associated antigen-specific anti-tumor response *in vivo* that is driven by GVHD. GVHD was identified as a driving force for GVT effects in RIST for solid tumors. It may represent one of the mechanisms contributing to GVT effects observed in allogeneic transplant recipients.

FUTURE DIRECTIONS

Control of Negative Aspects of RIST

Despite progressive improvement of transplant safety, the risk of significant transplant-related malignancy (TRM) limits the widespread application of allo-SCT for solid tumors. TRM remains 10–25% even in RIST. Without evidence of efficacy, most physicians considered this risk too high to justify studies of allo-SCT in patients with solid malignancies. The risk/benefit ratio is an important factor to decide the treatment plan in individual cases.

GVHD is the most significant concern in RIST as well as conventional allo-SCT (70). Approximately two-thirds of RIST recipients develop grade II–IV acute GVHD, and 10% of patients who receive RIST from an HLA-identical sibling died of GVHD in the National Cancer Center Hospital (70). Intensification of GVHD prophylaxis using potent immunosuppressive agents such as MMF, infliximab, ATG and CAMPATH-1H has contributed to improve GVHD-related outcomes (50,71,72); however, use of these agents might diminish GVT effects (50,68), and could increase the rate of serious infections (73). T-cell depletion can significantly reduce the risk of GVHD; however, it does not provide definite evidence of improving the outcomes of allo-SCT for solid or hematological malignancies. They might increase the risk of graft rejection and life-threatening infections (74). Several new strategies of T-cell depletion are currently under investigation, such as delayed T-cell add-back (75), the use of a suicide gene system (76), and selective $CD8^+$ depletion (77). Enhancement of the recovery of tissue damaged by GVHD is another promising approach. Some researchers showed that keratinocyte growth factor (KGF) administration is beneficial for the treatment and prevention of chemotherapy-induced gastrointestinal damage (78,79). It might ameliorate the organ damage caused by GVHD, leading to separation of GVHD from the beneficial GVL effects after allo-SCT (80). Since KGF has a possible risk of oncogenesis and cancer progression, further studies are required to investigate its safety in the setting of allo-SCT for solid tumors.

Another common immunological complication is the progression of the primary disease during immunosuppression. Preparative regimens of RIST have intense immunosuppressive effects to ensure the engraftment of donor cells. The half-life of antibodies such as ATG and CAMPATH-1H is so long as to maintain their immunosuppressive effects after RIST. Although these agents are effective in GVHD prophylaxis, they may deteriorate GVT effects and induce disease progression during immunosuppression (35). This phenomenon needs to be recognized as toxicity associated with conditioning regimens in RIST for solid tumors. However, when the primary disease is in progression at transplant, the possible association of conditioning regimens with early post-transplant progression cannot be distinguished from the natural course of the disease. This issue is troublesome in phase I or II clinical trials, particularly in solid tumors, as they are in progression at transplant. When the primary disease is in complete or partial remission, or stable disease at transplant, early post-transplant progression is more likely to be associated with conditioning regimens, requiring the clinician to be alert to this.

Enhancement of a GVT Reaction

Future studies should focus on directing the immune responses specifically to the tumors. In hematological malignancies, leukemia-specific cytotoxic T lymphocytes (CTLs) are frequently generated after allo-SCT, and are important in maintaining remission (81). Falkenburg et al. reported that treatment with *ex vivo*-generated leukemia-reactive T cells achieved remission in a patient with CML who relapsed after allo-SCT and was resistant to DLI (82). These results support the possibility of using DLI *ex vivo* primed against solid tumor cells. Several antigens targeted by alloreactive lymphocytes have been identified in allo-SCT for solid tumors. However, the expression of tumor-specific antigens varies considerably within the same tumor and at different stages of diseases. It is therefore difficult to produce antigen-specific CTLs in the treatment of solid tumors.

There are some possibilities to enhance tumor-specific allogeneic immunity prior to transplantation. One is to utilize donor cells activated against tumor alloantigens. While GVHD is a significant concern associated with pre-transplant immunization of allogeneic marrow donors with recipient-derived

tumor cells (83), some animal studies have shown that immunization of allo-SCT recipients with tumor cells can enhance GVT activities without exacerbating GVHD (84,85). It has been shown that CTLs can be generated using the whole tumor cells, which allows epitopes to be selected that are immunogeneic in the context of individual CTL repertoires (86). This approach can be applicable in allo-SCT for solid tumors with unknown target antigens. Morecki et al. reported that pre-immunization with mHa-mismatched tumor or spleen cells was capable of activating effector cells to induce GVT effects (87).

Post-transplant vaccination against tumor-specific or mHas or *ex vivo* generation of tumor-specific T cells followed by their adoptive transfer is another promising approach. Luznik et al. reported an animal model, showing a cooperation between host and donor T cells in the response to a tumor cell vaccine given after an RIST protocol that achieves stable mixed chimerism (88). GVT effects may be enhanced by the use of cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF), which may improve antigen presentation, and interferons, which may increase tumor antigen presentation by upregulating MHC class I and class II HLA molecules. Animal studies demonstrate that other cytokines such as IL-1 (89), IL-11 (90), and procedures capable of interfering with immunoregulatory mechanisms (91,92) are effective for inhibiting GVHD while preserving GVT effects.

Besides immunological approaches, it is critical to clarify the best timing and patient conditions for allo-SCT against solid tumors. Disease progression kinetic and immune status of the hosts are major factors influencing the sensitivity to allogeneic immunity (42). The efficacy of tumor cell eradication by alloreactive lymphocytes depends on the initial ratio between the number of tumor-specific immunocompetent cells in the graft and tumor cell burden of the recipient. Tumor debulking by the preparative regimen or surgical procedures before transplant might be important to enhance GVT effects. Preclinical evidence suggests that a lymphopenic host may represent a favorable clinical setting for immunotherapy (93). Dudley et al. provided evidence of cancer regression by the adoptive transfer of autologous tumor-reactive T cells directed against melanoma antigens in patients receiving a non-myeloablative, highly immunosuppressive preparative regimen (94). This approach may be helpful in allo-SCT for solid tumors.

Evaluation of Tumor Responses

Evaluation methods of tumor response to RIST have not been established. Even in the article of RIST for RCC by Childs et al. (35), their method of tumor response evaluation was not clearly described. It is critical to develop a global method to evaluate tumor response to RIST to share RIST results worldwide (95). Although the RECIST (Response Evaluation Criteria in Solid Tumors) system has been used as a gold standard to evaluate the response of solid tumors to treatment mainly in the field of cancer chemotherapy (96), it has not been fully validated in the area of allo-SCT for solid tumors. Compared with hematological malignancies, solid tumors are generally more resistant to the cytotoxic agents used in conditioning regimens administered before transplantation. Consequently, there may be some important differences in evaluating the response of solid tumors between RIST and conventional chemotherapy.

First, the feasibility of applying RECIST should be critically validated before its extensive application in transplantation (97). Tumor regression occurs several months after transplantation, and most tumors continue their natural growth until the manifestation of effective alloimmunity to restrain tumor growth. If the original RECIST criteria (96) are applied to patients undergoing RIST for solid tumors, most of the GVT effect would be evaluated as progressive disease, which would preclude subsequent evaluation (Fig. 3). Therefore, RECIST may underestimate the efficacy of RIST. Secondly, the proper time to measure the tumor size as a baseline for evaluating a subsequent tumor response has not been defined. In contrast to the results with chemotherapy, the tumor often temporarily increases in size following RIST. Accordingly, when the size at transplantation is used as a baseline, as in chemotherapy, a therapeutic effect following the initial progression could be overlooked or underestimated (Fig. 3). On the other hand, evaluating regression from the largest size after transplant certainly overestimates the effect of treatment (Fig. 3), and gives an unacceptable bias. Thirdly, the tumor size after RIST often fluctuates in response to a *de novo* GVT effect, post-transplant immunotherapy including DLI, and adjustment of immunosuppressive agents (Fig. 4). In this situation, it is clear that any evaluation of the response duration, such as progression-free survival and the overall response duration, is essentially impossible using the current RECIST criteria. Improved overall survival will ultimately be evaluated in phase III trials. To reach this point, a global standard evaluation system, that enables the

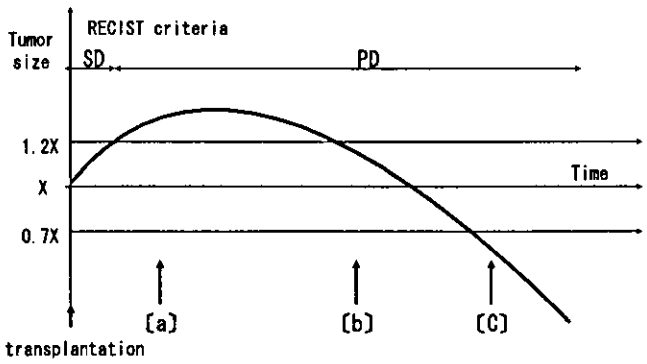

Figure 3. Course of tumor size after transplantation. Primary solid tumors are progressive despite chemoradiotherapy prior to transplantation. (a) Most tumors continue their natural growth until the development of a GVT effect, which usually occurs several months after transplantation. (b) If the tumor has increased in size compared with that at the time of transplant, regression from the largest size may overestimate the treatment effect. (c) If the tumor size at transplant is defined as a baseline, some treatment effects, observed in patients whose lesions show initial progression followed by regression with the development of GVHD, will be underestimated.

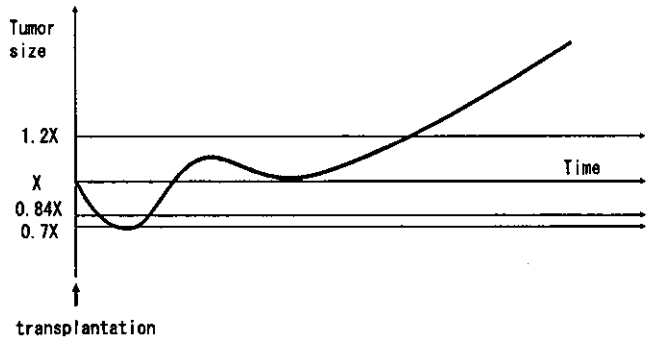

Figure 4. Fluctuation of tumor size after donor lymphocyte infusion or adjustment of immunosuppressive agents. It is difficult to handle patients in whom the tumor size fluctuates in response to post-transplant immunotherapy such as donor lymphocyte infusion and adjustment of immunosuppressive agents. Neither an appropriate timing of response evaluation nor an appropriate time to measure a baseline tumor size has been established in these cases.

effective screening of a therapeutic effect in an earlier phase II study, will need to be established. We hope that this review will inspire a productive discussion.

Use of Alternative Stem Cell Sources

Only 30–40% of patients in Japan have an HLA-identical sibling to serve as an allo-SCT donor. Unrelated bone marrow or umbilical cord blood may serve as an effective source of stem cells, thereby broadening the scope of patients who may benefit from allo-SCT. RIST using these stem cells is a promising alternative option. Some pilot studies have demonstrated the feasibility of allo-SCT from MUD (98,99) or using umbilical cord blood (100,101). Trials evaluating RIST using alternative stem cell sources have been started in many transplantation centers.

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Expression profiling and differential screening between hepatoblastomas and the corresponding normal livers: identification of high expression of the *PLK1* oncogene as a poor-prognostic indicator of hepatoblastomas

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Hepatoblastoma is one of the most common malignant liver tumors in young children. Recent evidences have suggested that the abnormalities in Wnt signaling pathway, as seen in frequent mutation of the *β-catenin* gene, may play a role in the genesis of hepatoblastoma. However, the precise mechanism to cause the tumor has been elusive. To identify novel hepatoblastoma-related genes for unveiling the molecular mechanism of the tumorigenesis, a large-scale cloning of cDNAs and differential screening of their expression between hepatoblastomas and the corresponding normal livers were performed. We constructed four full-length-enriched cDNA libraries using an oligo-capping method from the primary tissues which included two hepatoblastomas with high levels of alpha-fetoprotein (AFP), a hepatoblastoma without production of AFP, and a normal liver tissue corresponded to the tumor. Among the 10431 cDNAs randomly picked up and successfully sequenced, 847 (8.1%) were the genes with unknown function. Of interest, the expression profile among the two subsets of hepatoblastoma and a normal liver was extremely different. A semiquantitative RT–PCR analysis showed that 86 out of 1188 genes tested were differentially expressed between hepatoblastomas and the corresponding normal livers, but that only 11 of those were expressed at high levels in the tumors. Notably, *PLK1* oncogene was expressed at very high levels in hepatoblastomas as compared to the normal infant's livers. Quantitative real-time RT–PCR analysis for the *PLK1* mRNA levels in 74 primary hepatoblastomas and 29 corresponding nontumorous livers indicated that the patients with hepatoblastoma with high expression of *PLK1* represented significantly poorer outcome than those with its low expression (5-year survival rate: 55.9 vs 87.0%, respectively, $p = 0.042$), suggesting that the level of *PLK1* expression is a novel marker to predict the prognosis of hepatoblastoma. Thus, the differentially expressed genes we have identified may become a useful tool to develop new diagnostic as well as therapeutic strategies of hepatoblastoma.
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Introduction

Hepatoblastoma (HBL) is the most common hepatic cancer in children (Exelby *et al.*, 1975; Weinberg and Finegold, 1983). However, the etiology of HBL has been unclear in contrast to the adult hepatocellular carcinoma (HCC), in which preceding infection of hepatitis virus is often found (Buendia, 1992; Idilman *et al.*, 1998). Although most HBLs are sporadic, it is sometimes associated with certain hereditary diseases such as Beckwith–Wiedemann syndrome (Albrecht *et al.*, 1994) and familial adenomatous polyposis (Li *et al.*, 1987; Giardiello *et al.*, 1996; Kinzler and Vogelstein, 1996). In the former, loss of heterozygosity of chromosome 11p15.5 is frequently observed, and the abnormal regulation of the *insulin-like growth factor 2* (*IGF2*) and the *H19* genes at this locus may contribute to the disease (Albrecht *et al.*, 1994; Montagna *et al.*, 1994; Li *et al.*, 1995; Rainier *et al.*, 1995; Yun *et al.*, 1998; Fukuzawa *et al.*, 1999). In the latter, the *APC* gene, which is one of the key molecules in Wnt signaling, was found to be constitutively mutated (Kinzler and Vogelstein, 1996).

Increasing evidence suggests that Wnt signaling pathway also plays an important role in the genesis of sporadic hepatoblastomas. A high frequency (more than 60% in some reports) of somatic mutations in the *β-catenin* gene has recently been reported in sporadic tumors (Koch *et al.*, 1999; Wei *et al.*, 2000; Takayasu

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et al., 2001; Buendia, 2002). Mutant β-catenin proteins accumulate in the nucleus, resulting in stimulating transcription of the target genes such as *c-myc* and *cyclin D1* (Morin *et al.*, 1997; Polakis, 1999). Mutation in the *Axin* gene, whose product is an antagonist of nuclear accumulation of β-catenin, has also been found in HBL and may contribute to the pathogenesis of the tumors without *β-catenin* mutation (Taniguchi *et al.*, 2002; Miao *et al.*, 2003). However, the molecular mechanism underlying the pathogenesis of HBL is still largely unknown.

Recent progress in therapeutic strategies including intensive chemotherapy and liver transplantation improved the outcome of the patients with HBL. However, the prognosis of a significant fraction of the tumors still remains poor. The clinical markers currently used for HBL include staging, which is a major instrument for assessing prognosis (Hata, 1990), serum alpha-fetoprotein (AFP) (Mann *et al.*, 1978), mitotic activity (Haas *et al.*, 1989), DNA ploidy (Hata *et al.*, 1991), nuclear localization of β-catenin (Park *et al.*, 2001), p53 mutation (Oda *et al.*, 1995), and chromosomal alteration (Weber *et al.*, 2000). Serum AFP level is used as a diagnostic marker to monitor the tumor progression, responsiveness to the therapy, and recurrence after the treatment. Extremely high levels of serum AFP are reported to be associated with aggressiveness of the tumors with unfavorable outcome (van Tornout *et al.*, 1997), except some reports showing that there is no significant relationship between initial serum AFP levels and prognosis of the patients with HBL (Ortega *et al.*, 1991; von Schweinitz *et al.*, 1994). Moreover, the tumor with low levels of serum AFP often grows rapidly and is often reluctant to chemotherapy (von Schweinitz *et al.*, 1995). The other genetic markers including DNA ploidy, chromosomal aberration, and *p53* mutation are not so powerful clinical indicators. Even the nuclear localization of β-catenin and/or mutation of the *β-catenin* gene appear to lose their impact as a prognostic factor when combined with the grade of histological differentiation because of its close correlation with the latter (Takayasu *et al.*, 2001). Therefore, we may need to find novel markers to predict the patient's outcome in a comprehensive way.

To understand the molecular mechanism of the genesis and progression of HBL, as well as to develop a novel diagnostic and therapeutic system for the tumor, we have randomly cloned 10431 cDNAs expressed in primary HBL tissues and a normal infant's liver by using full-length-enriched oligo-capping cDNA libraries. In the present study, we have identified 86 genes differentially expressed between HBLs and their corresponding normal livers. One of such genes, *PLK1*, showed a significantly high expression in the formers as compared with the latters, and its high expression was significantly associated with poor prognosis of HBLs.

Results

Expression profiles of primary HBLs and a normal liver

To obtain the genes expressed in primary HBLs and normal infant's liver, we constructed oligo-capping cDNA libraries from two primary HBLs with increased AFP secretion (HMFT, HYST), a primary HBL without AFP secretion (HKMT), and a corresponding normal liver (HMFN). After cloning 3000 cDNAs from each of the four cDNA libraries, 2289, 2837, 2537, and 2768 clones from the libraries of HMFT, HYST, HKMT, and HMFN, respectively, were successfully end-sequenced. Homology search against the public databases of those 10431 clones by BLAST program revealed that 847 clones (8.1%) in total contained novel sequences which had not been annotated (Table 1).

To elucidate the gene expression pattern in each cDNA library, we compared expression profile of the known genes that appeared in three different kinds of libraries, a HBL with positive AFP (HMFT), a HBL with negative AFP (HKMT), and an infant's liver (HMFN) (Table 2). BodyMap (Okubo *et al.*, 1992) and a serial analysis of gene expression (SAGE) (Velculescu *et al.*, 2000) are very good methods to quickly provide quantification of the levels of all mRNAs in certain tissues and cell types by high throughput end-sequencing of cDNA clones. In this study, we applied the former method by counting cDNA clones to show each expression profile of HBL tumors or a non-tumorous tissue. Although each library consists of 3000 clones, which may be a rather small number, the frequency of each cDNA appearance provides a hint to understand each tissue's genetic background.

Overall, the most frequently appeared gene was *albumin* as expected, which was extremely low in the tumor with negative AFP. Genes involved in cellular structure and/or maintenance, glucose and lipid metabolisms, and a part of protein synthesis and its transport were frequently found in the normal liver library. On the

Table 1 Summary of the number of genes cloned from the cDNA libraries of hepatoblastomas and a normal infant liver of hepatoblastomas

Oligo-capping cDNA library	*No. of the clones*	*No. of the genes successfully end-sequenced*	*No. of the genes with unknown function*
Hepatoblastomas with positive AFP	6000	5126	323 (6.3%)
Hepatoblastoma with negative AFP	3000	2537	262 (10.3%)
Infant's liver	3000	2768	262 (9.5%)
Total	12000	10431	847 (8.1%)

Table 2 Comparison of the known genes frequently appeared in hepatoblastomas with or without secretion of AFP and a non-tumorous infant's liver

Gene symbol	*Acc. no.*	*Gene name*	*No. of appearance of the genes*		
			HBL with positive AFP	*Normal infant's liver*	*HBL with negative AFP*
Total number of genes			2289	2768	2537
Protein synthesis, metabolism, transport					
ALB	NM_000477	Albumin	558	482	8
AFP	NM_001134	Alpha-feto protein	67	0	0
AGT	NM_000029	Angiotensinogen	43	16	0
EEF1A1	X03558	Eukaryotic translation elongation factor 1 alpha 1	35	20	87
RPL27A	NM_000990	60S ribosomal protein L27a	31	4	52
FTL	M11147	Ferritin	24	11	3
FGA	NM_021871	Fibrinogen, A alpha polypeptide	20	38	2
HP	K01763	Haptoglobin	19	6	1
ORM1	X02544	Orosomucoid-1	12	8	0
RPS27	NM_001030	Ribosomal protein S27	11	4	31
F2	J00307	Coagulation factor 2	11	26	0
TF	NM_001063	Transferrin	8	6	0
PAH	U49897	Phenylalanine hydroxylase	6	6	0
PLG	NM_000301	Plasminogen	5	8	0
SERPINA1	X01683	Serine proteinase inhibitor, clade A, member 1	5	6	0
GC	NM_000583	Group-specific component	4	21	1
RPS29	NM_001032	Ribosomal protein S29	3	1	0
CTSB	NM_147783	Cathepsin B	2	5	3
SERPING1	BC011171	Serine proteinase inhibitor, clade G, menber 1	2	33	0
CRP	X56692	C-reactive protein	1	8	0
ITIH2	NM_002216	Inter-alpha (globulin) inhibitor, H2 polypeptide	0	25	0
Growth factor					
MST1	M74178	Macrophage stimulating 1	8	16	0
Cell signaling					
WIF1	NM_007191	Wnt inhibitory factor 1	0	0	11
DKK1	NM_012242	Dickkopf	0	0	7
Cell structure, adhesion					
VTN	NM_000638	Vitronectin	7	30	0
ACTB	BC013380	Actin	6	17	6
LRG	AF403428	Leucine-rich alpha-2-glycoprotein	6	11	0
VIM	NM_003380	Vimentin	0	3	38
Cell cycle					
RBM4	NM_002896	RNA binding motif protein	2	0	21
RAP1B	NM_015646	RAP1B	0	0	11
Organism defense					
BF	L15702	B-factor, properdin	5	13	0
GPX1	NM_000581	Glutathione peroxidase	4	0	0
C1R	NM_001733	Complement component 1	1	21	1
Glycometabolism					
LDHA	NM_005566	Lactate dehydrogenase	19	28	7
ADH1B	AF153821	Alcohol dehydrogenase	15	29	1
CES1	L07764	Carboxylesterase	9	22	2
ALDH1A1	NM_000689	Aldehyde dehydrogenase	2	13	2
Lipid metabolism					
EPHX1	NM_000120	Epoxide hydrolase 1	7	12	0
APOA2	NM_001643	Apolipoprotein A-II	6	2	0
ADFP	BC005127	Adipose differentiation-related protein	5	14	1
Heat shock protein, metabolic enzyme					
UGT2B4	Y00317	UDP-glucuronosyltransferase	11	32	2
HSPA8	NM_006597	Heat shock 70 kDa protein	1	6	1
Unknown, others					
ATP5A1	NM_004046	ATP synthase	18	11	23
SEPP1	NM_005410	Selenoprotein P	7	10	2

Table 2 (*continued*)

Gene symbol	*Acc. no.*	*Gene name*	*No. of appearance of the genes*		
			HBL with positive AFP	*Normal infant's liver*	*HBL with negative AFP*
CYP3A4	M18907	P450	6	81	3
AHSG	M16961	Alpha-2-HS-glycoprotein	6	5	2
TPT1	X16064	Translationally controlled tumor protein	6	0	3
CYP2C9	M61855	P4502C9	1	10	1

other hand, genes involved in protein synthesis such as elongation factors and ribosomal proteins were observed more frequently in HBLs than in normal liver. The expression profile in the library of the tumor without AFP secretion was very different from that with positive AFP (HMFT *vs* HKMT). As expected, *AFP* gene did not appear in the HKMT library. Intriguingly, *Wnt Inhibitory factor-1* and *dickkopf*, both of which are inhibitors of Wnt signaling (Hsieh *et al*., 1999; Wang *et al*., 2000), frequently appeared in the HKMT library. In addition, *vimentin*, *RNA-binding motif protein*, and *RAP1B* also frequently appeared in the HKMT library, but hardly in the HMFT library with AFP secretion. Thus, HBL with positive AFP and that with negative AFP seem to have a distinct gene expression profile, resulting in different biological characteristics.

Identification of the differentially expressed genes between HBLs and normal livers

To identify differentially expressed genes between HBLs and their corresponding normal livers, 1188 independent genes which included all of the 847 genes with unknown function and 341 known genes that were related to cellular functions including cell growth and differentiation among the 10431 cDNAs were selected and subjected to semiquantitative RT–PCR analysis (Figure 1a). The complementary DNAs reverse-transcribed from total RNA obtained from eight tumors and their corresponding normal livers were used as PCR templates after normalization with *GAPDH* expression. As a result, we found that 75 genes were expressed at higher levels in normal livers than in HBLs, whereas only 11 genes were expressed at higher levels in the tumors than in normal livers. Figure 1a shows the representatives of the results of differential screening using semi-quantitative RT–PCR and Table 3 lists 46 differentially expressed genes with known functions. We classified those differentially expressed genes into 12 categories according to their known functions. The genes preferentially expressed in normal liver showed the profiles which reflected normal liver function. Consistent with the previous reports about HBL and hepatocellular carcinoma (von Horn *et al*., 2001; Xu *et al*., 2001; Kinoshita and Miyata, 2002), *Insulin-like growth factor binding protein-3* (*IGFBP-3*), *aldolase B*, *ceruloplasmin*, and *c-reactive protein* were downregulated in HBLs as compared with the normal livers. The expression of *IGF2*, whose product has mitogenic

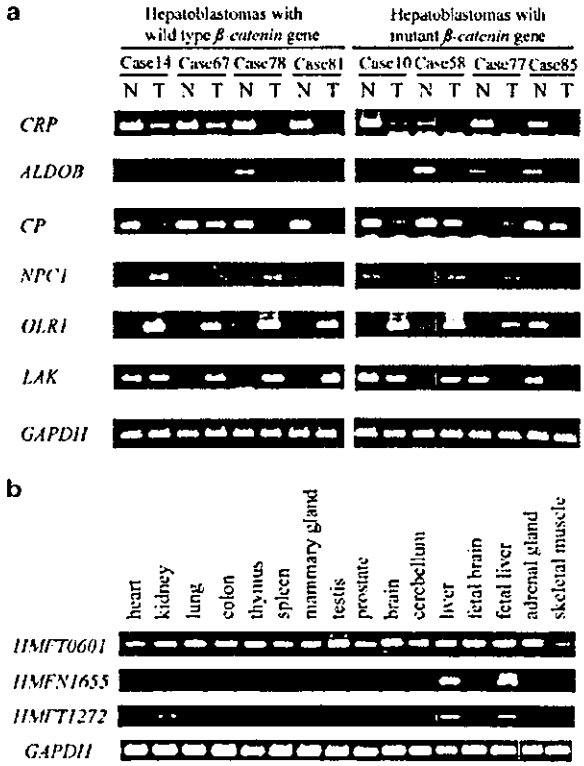

Figure 1 Expression of the representative genes by semi-quantitative RT–PCR. (**a**) Differentially expressed genes between HBLs with or without *β-catenin* mutation and the corresponding normal livers. cDNA was synthesized from RNAs prepared from eight pairs of tumors and their corresponding normal livers, and was used as a PCR template. Amount of cDNAs was normalized to that of *GAPDH*. Four tumors (cases 14, 67, 78, and 81) were with wild-type *β-catenin* gene, while the other four tumors (cases 10, 58, 77, and 85) were with mutant *β-catenin* gene. Gene symbols were shown on the left; *CRP*: C-reactive protein, *ALDOB*: aldolase, *CP*: ceruloplasmin, *NPC1*: Niemann–Pick disease, type C1, *OLR1*: oxidized low-density lipoprotein receptor 1, *LAK*: lymphocyte alpha-kinase. N: normal, T: tumor. (**b**) Semiquantitative RT–PCR of multiple human tissues. *HMFT0601* exhibited ubiquitous expression in all tissues examined, whereas *HMFN1655* and *HMFT1272* showed specific expression in liver and fetal liver

activity, is upregulated in HBLs, suggesting that the IGF axis may be involved in development of the tumor (Gray *et al*., 2000).

Four known genes which were expressed at high levels in HBLs (tumor > normal liver) include GTP-binding

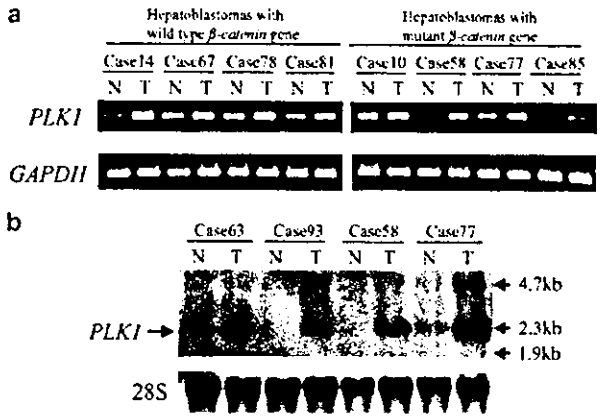

Figure 2 Increased expression of *PLK1* in HBLs. (a) Semiquantitative RT–PCR of *PLK1* gene in eight HBL cases. Preferential expression of the *PLK1* was seen in all sample pairs with and without *β-catenin* mutation. (b) Northern blot analysis of *PLK1* in primary HBLs, The 28S ribosomal band is shown as a control of each RNA amount

nuclear protein gene *RAN*, *PLK1* onocgene, and two cholesterol metabolism-associated protein genes, *low-density lipoprotein (LDL) receptor 1* and *Niemann-Pick disease type C1 (NPC1)*. The RAN protein is involved in the control of nucleo-cytoplasmic traffic of many nuclear proteins through formation of the transport nuclear pore complex (Ribbeck *et al.*, 1998). Nagata *et al.* (2003) also reported that *RAN* is upregulated in HBLs by oligonucleotide DNA array experiment. The LDL receptor 1 binds LDL, a major plasma cholesterol-carrying lipoprotein, and plays an important role in cholesterol homeostasis (Sudhof *et al.*, 1987; Goldstein and Brown, 1990; Hamanaka *et al.*, 1992). *NPC1* is a causal gene of Niemann–Pick type C disease which is an autosomal recessive lipid storage disorder that affects the viscera and central nervous system (Brady *et al.*, 1989). It encodes a protein with sequence similarity to the morphogen receptor 'patched', and to the cholesterol-sensing regions of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Loftus *et al.*, 1997) and is involved in the intracellular trafficking of cholesterol. Concerning the differentially expressed genes which contained unknown sequences, those cDNA sequences have been submitted to the public database (Genbank/DDBJ Accession numbers: AB07 3346-AB073347, AB073382-AB073387, AB073599-AB0 73614, and AB075869-AB075881). Interestingly, only one known gene, *lymphocyte alpha-kinase (LAK)*, showed distinct expression pattern between HBLs with mutant *β-catenin* and those with wild type *β-catenin* (Figure 1a).

We next examined expression pattern of the novel genes in human multiple tissues by semi-quantitative RT-PCR and found that at least five genes were specifically expressed in the liver (a part of the data is shown in Figure 1b). Since the oncogene *PLK1* (*polo-like kinase-1*) was expressed in HBLs at significantly high levels as compared with the corresponding normal livers, we further examined the role of its expression in HBL.

PLK1 oncogene is overexpressed in HBLs

Recent studies have demonstrated that the preferential expression of *PLK1* mRNA is associated with some cancers including non-small-cell lung cancer (Wolf *et al.*, 1997), squamous cell carcinoma of the head and neck (Knecht *et al.*, 1999), and esophageal carcinoma (Tokumitsu *et al.*, 1999). However, the role of *PLK1* in HBL has never been reported. As indicated by semi-quantitative RT-PCR described above, we found that *PLK1* mRNA expression in HBLs is higher than in normal livers (Figure 2a). Northern blot analysis also confirmed its higher expression in HBLs (Figure 2b). We also performed Southern blot analysis by using the genomic DNAs obtained from primary HBLs and human placenta as a control, and probed with the *PLK1*-specific DNA fragment. However, we failed to find any clue of rearrangements or amplification of the *PLK1* gene locus (data not shown).

To examine the clinical significance of the expression level of *PLK1*, we performed quantitative real-time RT–PCR analysis using 74 primary hepatoblastomas and 29 corresponding normal liver samples (Figure 3a). The average arbitrary values of *PLK1* expression in HBLs and normal livers were 28.9 ± 6.7 and 4.1 ± 0.76, respectively (mean $\pm$ s.e.m., $P < 0.01$). The average values in alive and dead cases were 21.7 ± 5.2 ($n = 61$) and 62.4 ± 28.2 ($n = 13$), respectively ($p = 0.021$). When we compared the expression levels of *PLK1* between 24-paired HBLs and their corresponding normal livers, the former in HBL samples was significantly higher in comparison with the latter ($P < 0.01$) (Figure 3b). We also examined the relationship between the expression levels of *PLK1* and clinicopathological data of HBLs. Statistically significant correlation was observed only between histology and *PLK1* expression ($p = 0.041$). The expression level of *PLK1* in the tumors with poorly differentiated histology was higher than those with the well-differentiated one. The other clinicopathological factors such as age, clinical stage, and *β-catenin* mutation did not show a statistical significance with *PLK1* expression.

To further examine whether the *PLK1* expression was associated with the outcome of the patients with HBL, we performed a Kaplan–Meier analysis (Figure 4). The distinction between high and low levels of *PLK1* expression was based on the median value (low, *PLK1* < 13 d.u.; high, *PLK1* ≥ 13 d.u.). Since the overall survivals of 15 out of 74 cases were unknown, 59 cases were applied to the analysis. The 5-year survival rates of the groups with high and low *PLK1* expression were 55.9 and 87.0%, respectively ($P = 0.042$). The univariate analysis showed that both *PLK1* expression ($P = 0.015$) and histology ($P = 0.025$) have a significant prognostic importance (Table 4). The multivariate analysis demonstrated that *PLK1* expression was significantly related to survival, after controlling *β-catenin* mutation, age, stage,

Table 3 The known genes differentially expressed between hepatoblastomas and normal livers

	Gene symbol	*Acc. no*	*Gene name*
Protein synthesis, metabolism, transport			
T>N	RAN	NM_006325	GTP-binding nuclear protein RAN
N>T	LBP	AF105067	Lipopolysaccharide-binding protein
N>T	TDO2	BC005355	Tryptophan 2,3-dioxygenase
N>T	CRP	X56692	C-reactive protein
N>T	GC	NM_000583	Group-specific component
N>T	HP	K01763	Haptoglobin
N>T	HPX	NM_000613	Hemopexin
N>T	SQSTM1	NM_003900	Sequestosome 1
N>T	PHDGH	AF171237	A2-53-73 3-phosphoglycerate dehydrogenase
N>T	PPP1R3C	XM_005398	Protein phosphatase 1, regulatory (inhibitor) subunit 3C
N>T	ITIH4	D38595	Inter-alpha-trypsin inhibitor family heavy chain-related protein
N>T	G1P2	M13755	Interferon-induced 17-kDa/15-kDa protein
Cytokine, growth factor, hormones			
N>T	HABP2	D49742	Hyaluronan binding protein 2
N>T	IGFBP3	NM_000598	Insulin-like growth factor binding protein 3
N>T	GOT1	AF052153	Glutamic-oxaloacetic transaminase 1
Cell signaling			
N>T	CSNK2B	M30448	Casein kinase II, beta polypeptide
N>T	TPD52	NM_005079	Tumor protein D52
cell cycle			
T>N	PLK1	X73458	PLK1
Cell structure, adhesion			
N>T	LRG	AF403428	Leucine-rich alpha-2-glycoprotein
N>T	PGRP-L	AF384856	Peptidoglycan recognition protein L precursor
N>T	CLDN4	NM_001305	Claudin4
N>T	VTN	NM_000638	Vitronection
Organism defense			
N>T	RODH-4	NM_003708	Retinol dehydrogenase 4
N>T	MASP1	AF284421	Mannan-binding lectin serine protease 1
N>T	C4BPA	M31452	Complement component 4 binding protein, alpha
Glycometabolism			
N>T	ADH1B	AF153821	Alcohol dehydrogenase 1B, beta polypeptide
N>T	ALDOB	M15657	Aldolase B
Lipid metabolism			
T>N	NPC1	NM_000271	Niemann–Pick disease, type C1
T>N	OLR1	NM_002543	Oxidized low density lipoprotein (lectin-like) receptor 1
N>T	DGAT2	AF384161	Diacylglycerol acyltransferase
N>T	SCP2	NM_002979	Sterol carrier protein 2
N>T	APOA5	AF202890	Apolipoprotein A-V
N>T	AADAC	L32179	Arylacetamide deacetylase
N>T	SAA4	M81349	Amyloid A protein
Transcription			
N>T	BZW1	NM_014670	Basic leucine zipper and W2 domains 1
N>T	CREB-H	NM_032607	CREB/ATF family transcription factor
RNA biogenesis, metabolism			
N>T	HNRPDL	AB017018	Heterogeneous nuclear ribonucleoprotein D-like
Homeostasis, heat shock protein, metabolic enzymes			
N>T	UGT1A	AF297093	UGT1 gene locus
N>T	ALPL	X14174	Liver-type alkaline phosphatase
N>T	SLC10A1	L21893	Solute carrier family 10
N>T	CES1	AF177775	Carboxylestelase
N>T	AKR1D1	Z28339	Aldo-keto reductase family 1, member D1
N>T	AKR1C2	U05598	Aldo-keto reductase family 1, member C2
N>T	CP	D45045	Ceruloplasmin
Others			
N>T	DGCR6L	NM_033257	DiGeorge syndrome critical region gene 6 like
N>T	A1BG	AF414429	Alpha-1-B glycoprotein

T>N: highly expressed in the tumors as compared to normal livers. N>T: highly expressed in normal livers as compared to the tumors