a larger patient population would clarify the influence of FDG PET/CT on sampling.

In our study, lymph node sampling was not performed in all patients because lymph nodes suspicious for metastasis in 9 of 15 patients (60%) were not accessible. In these lymph nodes, nodal staging was confirmed by an obvious progression in number and/or size of the lesions on follow-up examinations. This might be sampling bias in the statistical analysis.

In summary, the use of FDG PET/CT in patients with rhabdomyosarcoma increases the accuracy of overall staging and M staging compared to conventional staging. Our study suggests that whole-body FDG PET/CT should be the preferred modality with greater diagnostic accuracy for staging and re-staging in patients with rhabdomyosarcoma.

Acknowledgments The authors greatly thank for their assistance; Nagara Tamaki, MD, Noboru Oriuchi, MD, Keigo Endo, MD, Hiroshi Fujii, MD, Michio Senda, MD, Takashi Terauchi, MD, in the Scientific Research Expenses for Health and Welfare Programs, No. 17–12, the promotion and standardization of diagnostic accuracy in PET/CT imaging. This work was supported in part by grants from Scientific Research Expenses for Health and Welfare Programs, No. 17–12, the promotion and standardization of diagnostic accuracy in PET/CT imaging.

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VOLUME 27 · NUMBER 2 · JANUARY 10 2009

JOURNAL OF CLINICAL ONCOLOGY

SPECIAL ARTICLE

The International Neuroblastoma Risk Group (INRG) Staging System: An INRG Task Force Report

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Submitted February 18, 2008; accepted August 6, 2008; published online ahead of print at www.jco.org on December 1, 2008.

Supported by the William Guy Forbeck Research Foundation and Little Heroes Pediatric Cancer Research Fund; and Cancer Research UK and NHS funding to the NIHR Biomedical Research Centre (to A.D.J.P.).

Presented in part at the Advances in Neuroblastoma Research 12th Conference, May 17-20, 2006, Los Angeles, CA, and the International Society of Paediatric Oncology 38th Congress, September 18-21, 2006, Geneva, Switzerland.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

0732-183X/09/2702-298/$20.00

DOI: 10.1200/JCO.2008.16.6876

ABSTRACT

Purpose

The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pretreatment risk stratification. Because the International Neuroblastoma Staging System (INSS) is a postsurgical staging system, a new clinical staging system was required for the INRG pretreatment risk classification system.

Methods

To stage patients before any treatment, the INRG Task Force, consisting of neuroblastoma experts from Australia/New Zealand, China, Europe, Japan, and North America, developed a new INRG staging system (INRGSS) based on clinical criteria and image-defined risk factors (IDRFs). To investigate the impact of IDRFs on outcome, survival analyses were performed on 661 European patients with INSS stages 1, 2, or 3 disease for whom IDRFs were known.

Results

In the INGRSS, locoregional tumors are staged L1 or L2 based on the absence or presence of one or more of 20 IDRFs, respectively. Metastatic tumors are defined as stage M, except for stage MS, in which metastases are confined to the skin, liver, and/or bone marrow in children younger than 18 months of age. Within the 661-patient cohort, IDRFs were present (ie, stage L2) in 21% of patients with stage 1, 45% of patients with stage 2, and 94% of patients with stage 3 disease. Patients with INRGSS stage L2 disease had significantly lower 5-year event-free survival than those with INRGSS stage L1 disease (78% ± 4% *v* 90% ± 3%; $P = .0010$).

Conclusion

Use of the new staging (INRGSS) and risk classification (INRG) of neuroblastoma will greatly facilitate the comparison of risk-based clinical trials conducted in different regions of the world.

J Clin Oncol 27:298-303. © 2008 by American Society of Clinical Oncology

INTRODUCTION

The International Neuroblastoma Risk Group (INRG) classification system was developed to facilitate the comparison of risk-based clinical trials conducted in different regions of the world by defining homogenous pretreatment patient cohorts. As described in the companion article by Cohn and Pearson et al,[1] the INRG classification system was based on survival tree regression analyses of data collected on 8,800 patients. Because the International Neuroblastoma Staging System (INSS) stage of locoregional tumors is based on the degree of surgical resection, this staging system is not suitable for the INRG pretreatment risk classification system. Therefore, the INRG Task Force[1] (see Appendix, online only, for participants) developed a new staging system based on tumor imaging rather than extent of surgical resection.

The INSS was developed in 1986 after a meeting that was held to establish international consensus for a common staging system and response to therapy.[2,3] Although many countries around the world adopted the INSS, difficulties have been encountered. For example, according to the INSS, the same tumor can be either stage 1 or 3 depending on the extent of surgical excision, making direct comparison of clinical trials based on INSS stages difficult.[4] Furthermore, patients with localized disease who are observed because tumor regression is anticipated cannot be properly staged using INSS criteria.[5] An additional limitation of the INSS is that assessment of lymph node involvement is necessary for proper staging. However, lymph node sampling is subject to the

thoroughness of the individual surgeon, and the assessment of extra-regional lymph node involvement is difficult to apply uniformly.[2-4]

METHODS

Image-Defined Risk Factors

Since 1994, the International Society of Pediatric Oncology Europe Neuroblastoma Group (SIOPEN) has classified locoregional tumors as resectable or unresectable dependent on the absence or presence of "surgical risk factors," but independent of INSS stage.[6] Surgical risk factors are features detected on imaging that make safe, total tumor excision impracticable at the time of diagnosis.[6,7] The SIOPEN principle for stratifying patients with locoregional tumors by imaging features was adopted by the INRG Task Force at a conference in Whistler, Canada, in 2005, and used in the design of the INRG Staging System (INRGSS). However, to avoid confusion with the INSS, the terms resectable and unresectable are not used in the INRG system.

The premise is that a staging system based on preoperative, diagnostic images will be more robust and reproducible than one based on operative findings and approach. Furthermore, because digital radiographs can be reviewed centrally, the images can be evaluated uniformly. As the surgical risk factors are based on radiographic images, it was decided to use the term "image-defined risk factors" (IDRFs), and consensus was reached for the IDRFs listed in Table 1. The IDRFs and the INRGSS are designed for use at the time of diagnosis, but they may also be used at reassessments during treatment. Although not needed for staging patients with disseminated disease, it is recommended that the IDRF status of the primary tumor be recorded in all patients (including patients with metastatic disease), so that the impact of IDRFs on surgical resection, surgical complications, and outcome can be prospectively evaluated in all patients.

Table 1. Image-Defined Risk Factors in Neuroblastic Tumors

Ipsilateral tumor extension within two body compartments
Neck-chest, chest-abdomen, abdomen-pelvis
Neck
Tumor encasing carotid and/or vertebral artery and/or internal jugular vein
Tumor extending to base of skull
Tumor compressing the trachea
Cervico-thoracic junction
Tumor encasing brachial plexus roots
Tumor encasing subclavian vessels and/or vertebral and/or carotid artery
Tumor compressing the trachea
Thorax
Tumor encasing the aorta and/or major branches
Tumor compressing the trachea and/or principal bronchi
Lower mediastinal tumor, infiltrating the costo-vertebral junction between T9 and T12
Thoraco-abdominal
Tumor encasing the aorta and/or vena cava
Abdomen/pelvis
Tumor infiltrating the porta hepatis and/or the hepatoduodenal ligament
Tumor encasing branches of the superior mesenteric artery at the mesenteric root
Tumor encasing the origin of the coeliac axis, and/or of the superior mesenteric artery
Tumor invading one or both renal pedicles
Tumor encasing the aorta and/or vena cava
Tumor encasing the iliac vessels
Pelvic tumor crossing the sciatic notch
Intraspinal tumor extension whatever the location provided that:
More than one third of the spinal canal in the axial plane is invaded and/or the perimedullary leptomeningeal spaces are not visible and/or the spinal cord signal is abnormal
Infiltration of adjacent organs/structures
Pericardium, diaphragm, kidney, liver, duodeno-pancreatic block, and mesentery
Conditions to be recorded, but *not* considered IDRFs
Multifocal primary tumors
Pleural effusion, with or without malignant cells
Ascites, with or without malignant cells

Abbreviation: IDRFs, image-defined risk factors.

Staging Investigations

Diagnosis. In the INRG classification system, the diagnosis of neuroblastoma will be made using INSS criteria.[3] A tissue diagnosis of neuroblastoma can be made by conventional histology (with or without immunohistology and increased urine or serum catecholamine or metabolites). A diagnosis can also be made if unequivocal tumor cells are seen in bone marrow samples and increased urine or serum catecholamines or metabolites are present.

Involvement of bone marrow. Bone marrow involvement should be assessed by two aspirates and two biopsies from bilateral sites according to the recommendations of the INSS.[3] Biopsy is not required for infants younger than 6 months of age. Bone marrow disease is determined by morphology on smears and biopsies. Biopsies should be complemented by immunohistochemical techniques. Immunocytologic and/or molecular techniques are also recommended to evaluate the presence of tumor cells in the bone marrow at the time of diagnosis, although the results of these assays are not used for staging (Beiske et al, manuscript in preparation on behalf of the INRG Task Force).

Required imaging studies. Computed tomography (CT) and/or magnetic resonance imaging (MRI) with three-dimensional measurements and of sufficient quality to address IDRFs is mandatory for imaging the primary tumor. The presence or absence of each individual IDRF should be evaluated and recorded (Table 1). When possible, metastatic sites should also be measured by CT and/or MRI, as this information may be needed to evaluate treatment response.

Iodine-123 metaiodobenzylguanidine (MIBG) scintigraphy is mandatory, and it is recommended that the study is performed before tumor excision and according to the Standard Operating Procedure previously described.[8] One unequivocal MIBG-positive lesion at a distant site is sufficient to define metastatic disease. A single equivocal lesion on MIBG requires confirmation by another imaging modality (plain radiographs, and if negative, MRI) and/or biopsy.

Technetium-99 bone scintigraphy is required only exceptionally, but in all age groups, if MIBG positivity of the primary tumor cannot be confirmed (ie, the primary tumor is removed or is not MIBG-avid). An isolated bone uptake should be confirmed by another imaging modality and/or biopsy.

Staging Definitions

The short-version definitions of the four INRGSS stages are listed in Table 2.

Table 2. International Neuroblastoma Risk Group Staging System

Stage	Description
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
L2	Locoregional tumor with presence of one or more image-defined risk factors
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow

NOTE. See text for detailed criteria. Patients with multifocal primary tumors should be staged according to the greatest extent of disease as defined in the table.

Stage L1 tumors are localized tumors that do not involve vital structures as defined by the list of IDRFs (Table 1). The tumor must be confined within one body compartment, neck, chest, abdomen, or pelvis. The isolated finding of intraspinal tumor extension that does not fulfill the criteria for an IDRF (Table 1) is consistent with stage L1.

Stage L2 tumors are locoregional tumors with one or more IDRFs. The tumor may be ipsilaterally continuous within body compartments (ie, a left-sided abdominal tumor with left-sided chest involvement should be considered stage L2). However, a clearly left-sided abdominal tumor with right-sided chest (or vice versa) involvement is defined as metastatic disease.

Stage M is defined as distant metastatic disease (ie, not contiguous with the primary tumor) except as defined for MS. Nonregional (distant) lymph node involvement is metastatic disease. However, an upper abdominal tumor with enlarged lower mediastinal nodes or a pelvic tumor with inguinal lymph node involvement is considered locoregional disease. Ascites and a pleural effusion, even with malignant cells, do not constitute metastatic disease unless they are remote from the body compartment of the primary tumor.

Stage MS is metastatic disease in patients younger than 18 months (547 days) with metastases confined to skin, liver, and/or bone marrow. Bone marrow involvement should be limited to less than 10% of total nucleated cells on smears or biopsy. MIBG scintigraphy must be negative in bone and bone marrow. Provided there is MIBG uptake in the primary tumor, bone scans are not required. The primary tumor can be L1 or L2 and there is no restriction regarding crossing or infiltration of the midline.

Special Conditions

In addition to the IDRFs, and independent of the patient's INRGSS stage, three special conditions should be recorded: multifocal primary tumors, pleural effusion, and ascites (Table 1). Patients with multifocal primary tumors should be staged according to the greatest extent of disease as defined above (ie, stage L1, L2, M, or MS).

Relationship of INSS and INRG Stage

The INSS system is not in keeping with the INRG goal of a pretreatment classification system because the INSS assessment is made after the completion of the initial surgical procedure, and the INSS assessment is strongly dependent on the approach of the individual surgeon. To address these limitations, the INRGSS was developed. However, the survival tree regression analysis that forms the basis for the INRG classification system[1] could not be performed in terms of INRGSS because the sample size of patients with known surgical risk factors (analogous to the IDRFs that define INRGSS) in the INRG database[1] (< 850) was too small relative to patients with known INSS stage (> 8,500). Posthoc statistical analyses were therefore performed to determine whether it was reasonable to assign staging in terms of IDRFs of INRGSS instead of INSS, and if the prognostic ability of clinical stage was preserved if INRGSS was used. The analyses were restricted to patients with INSS stages 1, 2, or 3 disease because by definition, INSS stage 4 is equivalent to INRGSS M, and INSS stage 4S is very similar to INRGSS MS. Simon et al[9] have previously demonstrated the prognostic value of using IDRFs to define stage in a retrospective review of German neuroblastoma studies. The only other available data that can be used to validate the clinical significance of IDRFs and the INRGSS are those from SIOPEN in the INRG database.[1] The posthoc analysis of the SIOPEN data was performed in an attempt to validate the findings of the German study.

Statistical Considerations

Cross-tabulation of INRGSS and INSS was performed. The primary analytic end point for the predictive ability of INRGSS was event-free survival (EFS). Time to event was defined as time from diagnosis until time of first occurrence of relapse, progression, secondary malignancy, or death, or until time of last contact if none of these occurred. Univariate analyses were performed to assess the prognostic ability of INRGSS. Kaplan-Meier curves were generated, and curves were compared using log-rank test, with P values less than .05 considered statistically significant.[10] EFS and overall survival (OS) values were reported at the 5-year time point ± SE (per Peto).[11] It was not the goal of this analysis to compare outcome for INRGSS versus INSS (as was done in the study of Simon et al[9]).

Table 3. Distribution of SIOPEN Patients by INRGSS Versus INSS

INSS Stage	INRGSS L1		INRGSS L2		
	No.	%	No.	%	Total No.
1	239	79	64	21	303
2	81	55	66	45	147
3	12	6	199	94	211
Total	332	50	329	50	661

Abbreviations: SIOPEN, International Society of Pediatric Oncology Europe Neuroblastoma Group; INRGSS, International Neuroblastoma Risk Group Staging System; INSS, International Neuroblastoma Staging System.

RESULTS

A total of 661 patients with INSS stage 1, 2, and 3 disease from SIOPEN met INRG eligibility criteria and had known data for IDRFs. Twenty-one percent of patients with INSS stage 1, 45% of patients with INSS stage 2, and 94% of patients with INSS stage 3 disease had IDRFs (ie, in total, 50% of all localized tumors were INRGSS stage L2; Table 3). The remainder of patients who had no IDRFs were classified as having INRGSS stage L1 disease. Of the 661 SIOPEN patients, 474 patients had available outcome data. Both INSS and INRGSS were found to be highly prognostic. The EFS for patients with INRGSS stage L1 disease (90% ± 3%, n = 213) was statistically significantly higher than for stage L2 (78% ± 4%, n = 261; P = .0010; Fig 1). The OS for patients with INRGSS stage L1 disease (96% ± 2%) was also significantly higher than for patients with INRGSS stage L2 disease (89% ± 3%; P = .0068; Fig 2). The EFS for patients with INSS stage 1 disease (92% ± 3%, n = 209) was statistically significantly higher than for patients with INSS stage 2 (78% ± 6%, n = 103; P = .0005) and INSS stage 3 disease (75% ± 5%, n = 162; P < .0001), whereas patients with INSS stage 2 and 3 disease had similar EFS (P = .6611). The OS rates for patients with INSS stage 1, 2, and 3 disease were respectively 98% ± 2%, 95% ± 3%, and 84% ± 4%.

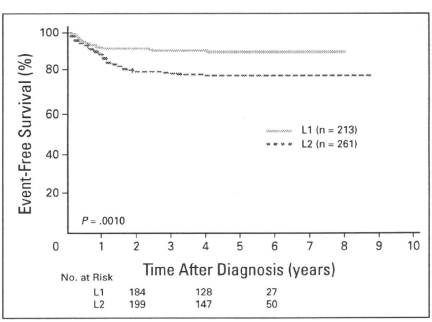

Fig 1. Event-free survival curves for International Society of Pediatric Oncology Europe Neuroblastoma Group patients by International Neuroblastoma Risk Group Staging System stage L1 versus L2 (P = .0010; n = 474). The number of patients at risk for an event are shown along the curves at years 2, 4, and 6.

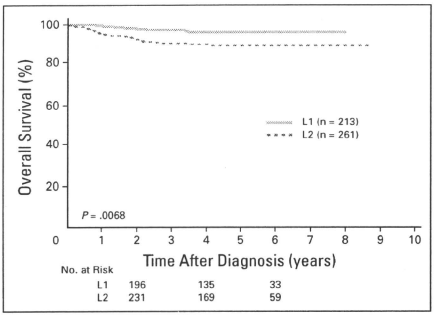

Fig 2. Overall survival curves for International Society of Pediatric Oncology Europe Neuroblastoma Group patients by International Neuroblastoma Risk Group Staging System stage L1 versus L2 ($P = .0068$; n = 474). The number of patients at risk for death are shown along the curves at years 2, 4, and 6.

DISCUSSION

Because excision of the primary tumor is a prerequisite for assigning patients to INSS stages 1 and 2, and because it is possible to downstage patients by surgical treatment at diagnosis,[4] the INSS is not suitable for pretreatment staging and risk assessment. A new clinical staging system (INRGSS) was, therefore, designed specifically to constitute one of seven prognostic factors in the INRG pretreatment classification system.[1] In the INRGSS, locoregional disease is stratified into two stages instead of three (as in INSS). This decision was based on recognition of the increasing importance of biologic prognostic factors and the excellent OS rate for patients with non-metastatic neuroblastomas.[1,12-16] Although the INRGSS can be used as a separate and independent clinical staging system, its primary function is as a component of the INRG. The INRGSS is not intended to substitute for the INSS, and it is anticipated that most cooperative groups will continue to use INSS in parallel with INRGSS.

Data from European studies show that absence or presence of IDRFs at diagnosis has prognostic significance. Our posthoc analysis of SIOPEN data[6] confirmed the results of Simon et al.[9] In both studies, EFS was lower for patients with INRGSS stage L2 compared with L1 tumors, and the differences were highly statistically significant. These observations support the translation of EFS tree regression results (in terms of INSS stages) into the INRG classification system (in terms of INRGSS): INSS 1 → INRGSS L1; INSS 2 and 3 → INRGSS L2; INSS 4 → INRGSS M; and INSS 4S → INRGSS MS.[1]

Because the treatment effect of tumor excision is an inherent part of the INSS, the prognostic value of specific stages within INRGSS and INSS cannot be directly compared. For example, most readers would agree that a comparison between patients with INRGSS stage L1 and INSS stage 1 is actually a comparison between an untreated group of patients and a cohort in whom nearly all patients have already been cured. However, even if INRGSS is not intended to substitute for the INSS, the distribution of patients between the two systems is of interest. In the retrospective study of Simon et al,[9] 84% of 160 patients with INSS stage 1 disease met the criteria for INRGSS stage L1 (ie, no IDRFs), whereas only 16% of 139 patients with IDRFs (stage L2) had INSS stage 1 disease. Similarly, our posthoc statistical analyses of 661 SIOPEN patients, in whom the clinical impact of surgical risk factors (= IDRFs) was examined prospectively, confirm the results of Simon et al.[9] In the data from SIOPEN (Table 3), 79% of patients with INSS stage 1 disease met the criteria for INRGSS stage L1, whereas 21% of patients with IDRFs (stage L2) had INSS stage 1 disease. In the SIOPEN LNESG1 study, 99% of 367 patients who met the criteria for INRGSS stage L1 underwent primary tumor excision (with one surgery-related death caused by renal failure). Among the 363 patients who underwent surgery, 75% had INSS stage 1 disease, 22% had INSS stage 2 disease, and 3% had INSS stage 3 disease. In 56% of 352 patients who had presence of one or more surgical risk factors (INRGSS stage L2), the initial surgical approach was limited to a biopsy; no attempt at primary tumor excision was made.[6] Furthermore, both studies referred to above demonstrated that primary operations in patients with IDRFs were associated with significantly lower complete excision rates and greater risks of surgery-related complications.[6,9]

Recommendations on treatment are not part of the INRGSS, nor of the INRG. Treatment policies must be decided by the individual cooperative groups. However, a new staging and risk classification system cannot exclude possible treatment alternatives, as is the case with INSS and the treatment option of observation without surgery. Today, OS in localized neuroblastoma is more than 90%,[1,12-16] and it can be assumed that a certain number of survivors have been overtreated. A main challenge in the years to come will be to maintain survival with reduced treatment. The INRGSS has been designed to permit uniform staging of all patients independent of the treatment alternatives contemplated.

The INRGSS differs from INSS in four important ways. First, it is based on preoperative imaging and IDRFs, not surgicopathologic findings. Second, the midline is not included in the staging criteria of the INRGSS. Third, lymph node status is not included in the staging of localized disease. Fourth, whereas INSS stage 4S has an upper age limit of 12 months, the Task Force decided to extend the age group for stage MS to patients younger than 18 months. The statistical basis for selecting a cutoff age of 18 months in INRG stages L2, M, and MS is presented and discussed in the companion article by Cohn and Pearson et al.[1] In one German study, the 5-year EFS was 100% in eight patients aged 12 to 18 months with *MYCN* nonamplified tumors who, apart from age, had classical INSS stage 4S disease.[17] The number of patients with "stage 4S disease aged 12 to 18 months" is small, but because the outcome in this patient cohort remains unclear, it is anticipated that the individual cooperative groups will give these patients special attention in prospective studies where careful stopping rules are included. Unlike INSS stage 4S, stage MS includes patients with primary tumors infiltrating the midline (INSS stage 3). The inclusion of all patients with stage L2 primaries is supported by the results of the SIOPEN 99.2 trial (B. De Bernardi, personal communication, February 2008). In this study, all 30 infants with INSS stage 4 disease having primary tumors corresponding to INSS stage 3 disease because of midline infiltration, and with stage 4S metastatic pattern, survived. Eight patients received no chemotherapy, and the remainder received only one or a few courses of chemotherapy to control symptoms. Only five of the patients had their primary tumor excised.

The effects of treatment on IDRFs are not known, although preliminary data from the SIOPEN Infant Neuroblastoma Study suggests that preoperative chemotherapy (or time) can decrease the incidence of IDRFs by 35% to 40%.[18] It also remains unclear whether the risks of surgical complications are reduced by preoperative chemotherapy when delayed operations are performed in patients who have persistent IDRFs. The impact of individual IDRFs on outcome is currently not known, and the clinical significance of individual IDRFs will need to be analyzed in a larger series of patients to address these questions.

Although surgery is not required for INRGSS staging, the biologic characteristics of the tumor must be known to stratify patients according to the INRG pretreatment classification system.[1] Image-guided core-needle biopsies are acceptable provided adequate material for the histologic and genetic studies are obtained. However, in many cases, complete or partial tumor excision may be a more rational way to obtain tissue for histologic categorization and genetic studies. In the latter case, it must be emphasized that the magnitude of the residual tumor does not influence the INRG stage. Even if completely excised at diagnosis, a localized tumor with (preoperative) one or more IDRFs will still be classified as an INRGSS stage L2.

The Task Force considered using a specific nomenclature to identify subgroups of patients with neuroblastoma with special features like multifocal primary tumors (because of the potential genetic implications of this diagnosis[19,20]). The experience with the INSS does not support a practice of subclassification within a staging system. Although the stage of patients with multifocal primary tumors in the INSS should be given a subscript letter M (stage 1_M, stage $2A_M$, and so on),[3] this subscript has not been widely accepted and only rarely used in published series. The Task Force, therefore, decided not to use subscripts in the INRGSS. This decision implies that patients with important special features not defined by the INRGSS have to be identified by other measures. It is recommended that data regarding the conditions listed in the last portion of Table 1 be collected.

Isolated pleural effusion and ascites are not considered IDRFs in the INRGSS. Although pleural disease is associated with reduced survival rates in patients with metastatic neuroblastoma,[21,22] isolated pleural effusion or ascites is rare in patients with locoregional disease, and its impact on outcome is not clear. In a recent study of 31 patients with neuroblastoma having pleural effusion, none had INSS stage 1 disease and only one had stage 2 disease.[23] It is assumed that the vast majority of patients with ascites also have either metastatic disease or the presence of IDRFs.

The extent of intraspinal tumor extension can range from a small tumor component bulging through one intervertebral foramen to a tumor occupying the majority of the spinal canal. In the SIOPEN studies, intraspinal tumor extension is considered a surgical risk factor if neurologic signs of spinal cord compression are present. However, because clinical signs are not image defined, in INRGSS, it was decided to consider intraspinal tumor extension an IDRF, provided one or more of the imaging criteria listed in Table 1 are present.

In conclusion, the INRGSS is a preoperative staging system that has been developed specifically for the INRG classification system. The extent of disease is determined by the presence or absence of IDRFs and/or metastatic tumor at the time of diagnosis, before any treatment. Use of this pretreatment staging system and the INRG classification system will facilitate the ability to compare results of risk-based clinical trials conducted in different regions of the world, and thereby, provide insight into optimal treatment strategies for patients with neuroblastic tumors.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Acknowledgment

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LIVER TRANSPLANTATION 14:1718-1724, 2008

ORIGINAL ARTICLE

Liver Regeneration in Donors and Adult Recipients After Living Donor Liver Transplantation

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In living donor liver transplantation, the safety of the donor operation is the highest priority. The introduction of the right lobe graft was late because of concerns about donor safety. We investigated donor liver regeneration by the types of resected segments as well as recipients to assess that appropriate regeneration was occurring. Eighty-seven donors were classified into 3 groups: left lateral section donors, left lobe donors, and right lobe donors. Forty-seven adult recipients were classified as either left or right lobe grafted recipients. Volumetry was retrospectively performed at 1 week, 1, 2, 3, and 6 months, and 1 year after the operation. In the right lobe donor group, the remnant liver volume was 45.4%, and it rapidly increased to 68.9% at 1 month and 89.8% at 6 months. At 6 months, the regeneration ratios were almost the same in all donor groups. The recipient liver volume increased rapidly until 2 months, exceeding the standard liver volume, and then gradually decreased to 90% of the standard liver volume. Livers of the right lobe donor group regenerated fastest in the donor groups, and the recipient liver regenerated faster than the donor liver. Analyzing liver regeneration many times with a large number of donors enabled us to understand the normal liver regeneration pattern. Although the donor livers did not reach their initial volume, the donors showed normal liver function at 1 year. The donors have returned to their normal daily activities. Donor hepatectomy, even right hepatectomy, can be safely performed with accurate preoperative volumetry and careful decision-making concerning graft-type selection. ***Liver Transpl 14:1718-1724, 2008.*** © **2008 AASLD.**

Received December 13, 2007; accepted July 19, 2008.

Living donor liver transplantation (LDLT) plays an important role because of cultural attitudes and the scarcity of cadaveric donations in Japan.[1] LDLT involves an ethical problem: because the donor operation is performed on a healthy person, donor safety is the highest priority and requires special attention. LDLT was a treatment first for infants, then for children, and finally for adults. Although a left lobe graft provides enough volume for pediatric recipients, some adult recipients require a right lobe graft. There is little question about donor safety, on the basis of remnant liver volume requirements alone, if only a left lateral section and left lobe graft are procured. It is in the use of a right lobe that issues of donor safety come into play. Several authors have studied donor safety, especially with respect to surgical complications and clinical courses.[2,3] Donor safety from various standpoints should be studied further in order to gain insight into the postoperative recovery pattern of a healthy person and to prevent complications.

Although the human liver can tolerate more than 70% hepatectomy,[4] unfortunately some living donors have died of excessive loss of the liver.[5-7] Precise evaluations of donor liver volume are important in order to prevent unexpected hepatic insufficiency and to evaluate normal liver regeneration, which is still unknown to a great extent. Several authors have studied liver regeneration, mostly in diseased livers.[8,9] Complete and

Abbreviations: CT, computed tomography; GW, graft weight; LD, left lobe donor; LDLT, living donor liver transplantation; LR, left lobe grafted recipient; LSD, left lateral section donor; RD, right lobe donor; RR, right lobe grafted recipient; SLV, standard liver volume.
This research was partially supported by the Ministry of Education, Culture, Sports, Science, and Technology through a Grant-in-Aid for the 21st Century Center of Excellence Program entitled "Establishment of Individualized Cancer Therapy Based on Comprehensive Development of Minimally Invasive and Innovative Therapeutic Methods (Keio University)."
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DOI 10.1002/lt.21622
Published online in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Profiles of Donors Who Participated in Postoperative Volumetry

Group	n	Male/Female	Age (Years)*	GW (g)†	Ratio of Volume Scheduled To Be Resected (%)‡
LSD	28	15/13	30.5 ± 4.8	247.1 ± 45.9	20.6 ± 5.0
LD	32	21/11	39.4 ± 13.9	442.5 ± 129.4	34.8 ± 9.6
RD	27	10/17	45.1 ± 11.7	627.7 ± 105.7	54.5 ± 10.2

NOTE: All values are expressed as mean ± standard deviation.
Abbreviations: GW, graft weight; LD, left lobe donor; LSD, left lateral section donor; RD, right lobe donor.
*$P < 0.05$ for LSD versus LD and RD.
†$P < 0.05$ for LSD versus LD and RD and for RD versus LD.
‡RD versus LSD and LD.

TABLE 2. Profiles of Recipients Who Participated in Postoperative Volumetry

Group	n	Male/Female	Age (Years)	Acquired GW (g)*	GW/SLV (%)*
LR	22	9/13	42.6 ± 12.1	470.6 ± 122.4	43.3 ± 10.2
RR	25	19/6	45.0 ± 12.7	639.9 ± 98.7	53.8 ± 9.7

NOTE: All values are expressed as mean ± standard deviation.
Abbreviations: GW, graft weight; LR, left lobe grafted recipient; RR, right lobe grafted recipient; SLV, standard liver volume.
*$P < 0.05$.

prompt liver regeneration occurs in donors and recipients in most circumstances.[10,11] It is clinically important to determine liver regeneration in donors as well as recipients. In the present study, liver regeneration in adult recipients was also evaluated for comparison with normal liver regeneration. Volumetric analysis was performed without consideration of the existence of postoperative complications or the recipient primary disease. Accurate reporting on surgical outcomes and liver regeneration helps to ensure proper informed consent, which is obtained from prospective donors.

Liver regeneration is one of the factors reflecting surgical stress and recovery. If a notable difference in the postoperative clinical course, mainly liver regeneration in this study, is not shown between left lateral section donors (LSDs), left lobe donors (LDs), and right lobe donors (RDs), it would mean that the operation for RDs, as well as that for LSDs and LDs, is safe. The purpose of this study was to evaluate liver regeneration in donor groups with respect to the types of resected segments and in recipients and ensure that appropriate regeneration was occurring in both donors and recipients.

PATIENTS AND METHODS

Patients

Between April 1995 and August 2005, 100 LDLTs were performed at Keio University Hospital. The remnant liver volume in donors and the graft volume in recipients were retrospectively measured by computed tomography (CT) films at 1 week, 1, 2, 3, and 6 months, and 1 year after the operation. Eighty-seven donors and 47 adult recipients were enrolled in this study. Patients without regular CT were excluded from this study. Pediatric recipients were excluded because liver regeneration was compared between donors and recipients who had almost the same body weight. Donors were classified into 3 groups: LSD (n = 28), LD (n = 32), and RD (n = 27). Donors underwent volumetric CT assessment of their livers before LDLT to evaluate the whole liver volume and to determine the graft type. Adult recipients were classified into 2 groups: left lobe grafted recipients (LRs; n = 22) and right lobe grafted recipients (RRs; n = 25). Because the calculation of the degree of liver regeneration was based on the standard liver volume (SLV), recipient SLV was calculated with the following equation: SLV = 706.2 × body surface area + 2.4.[12] The graft type was selected according to criteria described previously.[13] In summary, the graft weight (GW) is to be less than 65% of the donor whole liver volume (ie, the remnant liver volume is to be more than 35% of the donor whole liver volume), and GW is to be more than 35% of recipient SLV, which is enough to maintain sufficient liver function. Donor hepatectomy and recipient transplant procedures were performed as described previously.[14]

Donor and Recipient Profiles

Donor and recipient profiles are summarized in Tables 1 and 2, respectively. Donor and recipient ages ranged from 19 to 65 years and from 19 to 63 years, respectively. The average age in the LSD group was the lowest among the groups, and the individual age in that group ranged from 21 to 38 years. The individual ages in the LD and RD groups ranged from 19 to 65 years and from 21 to 65 years, respectively. No donor received a blood transfusion. The mean length of hospital stay after the

operation was 14.8 ± 7.8, 16.8 ± 8.7, and 15.9 ± 7.8 days in the LSD, LD, and RD groups, respectively. It did not differ significantly between the groups. Although complications prolonged hospital stays, the mortality rate (grade V complications of the Clavien classification)[15] among the donors was zero. The postoperative morbidity rate was 21.8% (n = 19). Major complications were bile leakage, wound infection, and fluid collection. Although 1 donor underwent an operation for wound infection (grade III), all other donors recovered completely with conservative treatment (grade II). All donors are currently alive and have returned to their normal daily activities. Among recipients, no significant age differences were found between the LR and RR groups. Ages ranged from 21 to 59 years and from 19 to 63 years in the LR and RR groups, respectively. The postoperative morbidity rate of recipients was 70.3% (grades II-IV), and the mortality rate (grade V) was 19.2%. Diverse recipient complications affected the length of hospital stay. The mortality and morbidity rates did not differ significantly between the groups.

Volumetric Analysis

The donor whole liver volume, donor remnant liver volume, and recipient grafted liver volume were measured by CT films.[16] In the volumetric study, serial transverse scans were performed from the dome to the most inferior portion of the liver. Each slice of the liver was outlined, and the edge of the region of the liver was traced. The liver images were then uploaded from an image scanner (Seiko Epson, Nagano, Japan) to a computer. The profile of the liver image was traced, and an image processing program [Scion Image (public domain software), Scion Corp., Maryland] calculated the liver area. The liver volume was finally calculated by integration of the images from each liver region. GW was measured immediately after retrieval of the graft. The specific gravity of a normal liver is about 1.0, so the volume is similar to the weight. The donor remnant liver volume (day 0) was calculated as follows: whole liver volume − GW. For all patients, we compared the measured actual liver volume postoperatively with the calculated liver volume to obtain the degree of liver regeneration. This value was expressed as a percentage.

Statistical Analysis

All measured values are expressed as the mean ± standard deviation. Statistical significance ($P < 0.05$) was examined by a paired t test. Statistical analysis was performed with SPSS 14.0 (SPSS, Inc., Chicago, IL).

RESULTS

Remnant Liver Volume and Graft Liver Weight

The actual GW ranged from 166 to 928.7 g. The average percentage of GW/donor whole liver volume was 54.5% even in the RD group. This percentage ranged from 13.7% to 37.4%, from 19.7% to 63.4%, and from 27.0% to 70.5% in the LSD, LD, and RD groups, respectively. Among all groups, GW/donor whole liver volume was more than 65% in only 2 cases, both of which were in the RD group. The percentages of GW/donor whole liver volume in these cases were 66.6% and 70.5%, but those at preoperative volumetry were 63.9% and 64.1%, respectively. The percentages of GW/recipient SLV ranged from 29.7% to 72.4% in the LR group and from 41.3% to 84.4% in the RR group. The GW/recipient SLV ratio was less than 35% in 4 cases, all of which were in the LR group. All 4 of these cases showed more than 35% GW/recipient SLV at preoperative volumetry. When right lobe grafts were transplanted, GW/donor whole liver volume was more than 65%, and in 3 cases, it exceeded 70%. A strong positive correlation ($r = 0.92$) was observed between the estimated graft volume and actual GW (Fig. 1).

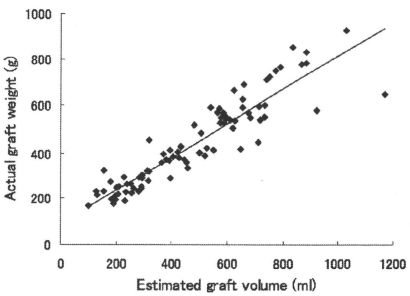

Figure 1. Correlation between the estimated graft volume and actual graft weight. A strong positive correlation was observed between the estimated graft volume and actual graft weight.

Liver Regeneration

Preoperative donor whole liver volume and recipient SLV were regarded as 100% to evaluate the patterns of postoperative liver regeneration, which are shown in Fig. 2. The remnant liver volume of the LSD group was significantly larger than those of the LD and RD groups. The average resected liver volume of the LSD group was about 20% of the whole liver volume. In the RD group, in contrast, the remnant liver volume was 45.4% of the whole liver volume immediately after the operation and then increased rapidly from 68.9% at 1 month to 89.8% at 6 months. At 6 months, the remnant liver volume in the RD group regenerated dramatically, and the regeneration rate was almost the same as those of the LSD and LD groups. In the LSD and LD groups, the liver volume increased gradually until 1 week and then decreased from 1 month to 3 months, reaching around 90% of the preoperative liver volume at 1 year. Among recipients, both the RR and LR groups showed the same regeneration pattern.

Figure 3 shows the patterns of liver regeneration in

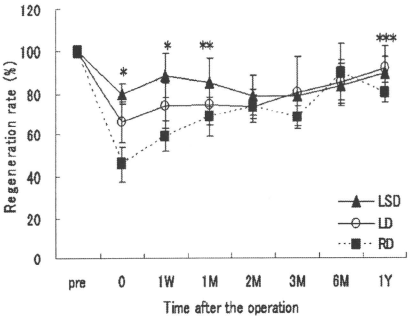

Figure 2. Changes in the remnant liver volume in donors. Rapid liver regeneration was observed in the RD group. A *P* value < 0.05 is shown as follows: *LSD versus LD and RD and LD versus RD, **LSD versus LD and RD, and ***LD versus RD. Abbreviations: LD, left lobe donor; LSD, left lateral section donor; RD, right lobe donor.

donors and recipients. The same liver segment of each regeneration pattern was evaluated between donors and recipients as follows: (1) the RD group, for which the remnant liver was the left lobe, and the LR group, which received a left lobe graft, and (2) the LD group, for which the remnant liver was the right lobe, and the RR group, which received a right lobe graft. Transplanted grafts regenerated rapidly immediately after LDLT until 2 months, exceeding SLV in both the LR and RR groups. After 2 months, the volume gradually decreased to 90% of SLV. On the other hand, the donor remnant liver volume tended not to reach the preoperative whole liver volume until 1 year. The recipient grafted liver volume increased more rapidly than the donor remnant liver volume, regardless of the type of liver segment.

In Fig. 4, the regeneration rates in donors and recipients who shared the same source of hepatocytes were evaluated as donor-recipient pairs. In this evaluation, the liver volume immediately after the operation, donor remnant liver volume, and recipient grafted liver volume were regarded as 100%. The average regeneration rates in the RD and RR groups were increased from 128.1% ± 20.1% and 165.8% ± 26.0% at 1 week to 159.9% ± 49.6% and 184.7% ± 36.9% at 1 month. The recipient liver regenerated faster than the donor liver. The same result was obtained in the LD and LR groups. The average regeneration rates in the LD and LR groups were increased from 118.0% ± 26.0% and 187.7% ± 32.1% at 1 week to 122.5% ± 24.6% and 200.6% ± 25.2% at 1 month. The less the liver volume was immediately after the operation, the faster the liver regenerated in both the donor and recipient groups.

DISCUSSION

Donor safety is the highest priority because the donor operation is performed on a healthy person in LDLT.

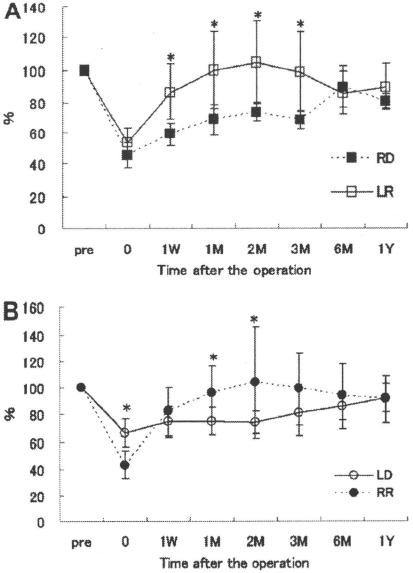

Figure 3. Patterns of liver regeneration in donors and recipients. (A) Donors whose remnant liver is the left lobe (RD) and recipients with a grafted left lobe (LR). (B) Donors whose remnant liver is the right lobe (LD) and recipients with a grafted right lobe (RR). The recipient liver regenerated faster than the donor liver. A *P* value < 0.05 between the donor and recipient is indicated by an asterisk. Abbreviations: LD, left lobe donor; LR, left lobe grafted recipient; RD, right lobe donor; RR, right lobe grafted recipient.

Therefore, the preoperative liver volume must be estimated accurately in order to avoid donor death resulting from excessive loss of the liver. LDLT using right lobe grafts was not performed until relatively recently out of concern for donor safety. At Keio University Hospital, adult-to-adult LDLT was started in June 1997, and right lobe graft was first performed in April 1999. A retrospective volumetric study can be performed here because long-term follow-up has been going on for a decade and a large number of LDLTs were performed after the first case of adult-to-adult LDLT.

The LSD group was comparatively young because lateral segment grafts were transplanted to pediatric recipients. These recipients acquired grafts from parents who were younger than the average age of the subjects in this study in most cases. On the other hand, grafts to adult recipients were from their children or elderly parents. This is why the average age of the RD group was high as right lobe grafts were transplanted to adult recipients. The average age differed significantly

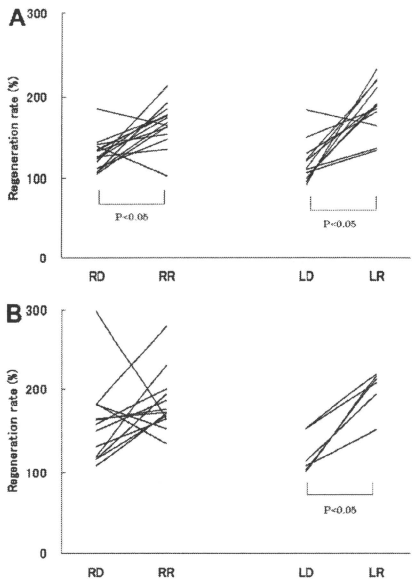

Figure 4. Liver regeneration ratio at (A) 1 week and (B) 1 month after living donor liver transplantation. The lines connect the donor and recipient who share the same source of hepatocytes. Liver volume immediately after the operation was regarded as 100%. In most cases, the regeneration ratio of the recipient liver was higher than that of the donor liver. Abbreviations: LD, left lobe donor; LR, left lobe grafted recipient; RD, right lobe donor; RR, right lobe grafted recipient.

within the donor group but not within the recipient group. Donor age and gender may affect liver regeneration. Some series have reported increased regeneration in younger donors and female donors, possibly secondary to estrogen.[17,18] However, some authors have reported that the liver regeneration rate does not differ by donor age[19] or gender.[20,21] The influences of donor age and gender-related differences on liver regeneration need to be evaluated further.

In this study, liver volume was evaluated by CT. CT is being used as a noninvasive method for evaluating liver vascular anatomy and estimating graft volume. Several types of errors can affect the measurement of liver volume. However, an excellent linear correlation was found between estimated graft volume and actual GW in our study. This means that most of the preoperative graft volume was estimated exactly. With this reliable method, the regeneration of donor remnant livers and that of recipient grafted livers were calculated. CT volumetry revealed that more than 65% of the whole liver volume was resected in 2 donors, and 4 recipients did not gain a graft liver that was more than 35% of their SLV. This implies that these donors and recipients may have insufficient volume to meet their metabolic needs according to our criteria. By preoperative volumetry, these donors and recipients were estimated to have not lost more than 65% of their whole liver volume or to have gained a graft liver less than 35% of their SLV. Thus, if a left lobe graft left the donor with enough liver volume, the recipient did not gain enough liver volume for his or her SLV. In contrast, if a right lobe graft was used to give a recipient sufficient liver volume for his or her SLV, the donor remnant liver volume was too small to meet his or her metabolic demand. For those donors, the liver regenerated sufficiently, and they did not experience liver failure.

The liver regenerates until the liver volume/body weight ratio plateaus. The liver regeneration process has been divided into 3 phases.[22] The early phase is rapid regeneration, which occurs during the first 2 postoperative weeks and is associated with vascular engorgement and tissue edema. The second phase is volume decline, which may be attributable partially to the normalization of developed vascular engorgement or tissue edema at 1 to 2 months after hepatectomy. The third phase is a slow increase in volume, which occurs again until the volume reaches a constant level. In the LSD and LD groups, livers regenerated according to this pattern. In the RD group, the remnant liver continued to regenerate until 2 months without decreasing volume, and volume decline was observed at 3 months. The smaller the remnant liver is, the longer the liver may continue to regenerate up to an extent enough to meet the metabolic needs, regardless of the high regeneration speed. Therefore, the volume decline in the RD group occurred later than those in the LSD and LD groups. Similarly to results published by Nadalin et al.,[23] the RD livers did not return to their full volume but still functioned well without graft failure and with normal liver function at 7 days after the operation. This suggests that livers can function well without returning to the initial volume.

We evaluated adult recipient liver regeneration to reveal how the donor liver regenerates after it was transplanted into recipient, diseased person. The donor and recipient shared the same normal liver parenchyma. Child recipients were excluded because their body size greatly differed from that of the donors. In donor-recipient pairs, the same segment was evaluated, and the source of hepatocytes was the same. The left lobe, which had almost the same liver volume percentage of the whole liver volume immediately after the operation, regenerated faster in recipients than in donors. On the other hand, although the liver volume percentage differed between donors and recipients, the same regeneration pattern observed in the left lobe was observed in the right lobe. The recipient grafted liver regenerated faster than the donor remnant liver regardless of the immediate postoperative volume. Rapid recipient liver regeneration may be related to high liver blood flow after LDLT because of persistence of a hyperdynamic state, immunosuppressant administration, or humoral

factors in the recipient.[10] Cyclosporine[24] and tacrolimus[25] stimulate liver regeneration.

In our previous study, the smaller the liver graft was with respect to the recipient body size and the higher the portal inflow was to it, the more rapidly the liver regenerated after LDLT.[26] Rapid liver regeneration occurred in the small remnant livers and grafts.[27] Several other factors affect liver regeneration, such as the middle hepatic vein,[28] portal venous flow,[26] spleen size,[21] and cytokines.[29,30] The liver regenerates faster with high portal venous flow and preservation of the middle hepatic vein. On the other hand, an enlarged spleen inhibits liver regeneration because an inhibitory protein was released from the spleen cells. Some of the most notable cytokines that induce liver regeneration are hepatocyte growth factor, interleukin-6, and tumor necrosis factor-α. In particular, hepatocyte growth factor plays an important role in liver regeneration. The decline of liver volume induced hepatocyte growth factor production, and liver regeneration was stimulated. Although liver regeneration was evaluated with the exclusion of these factors in the present study, further evaluation considering these factors should reveal in detail the mechanism underlying liver regeneration.

Our retrospective study contributes to our understanding of accurate patterns of normal liver regeneration, which were not evaluated in a previous volumetric study. In this study, liver regeneration was evaluated many times from the preoperative day to 1 year after the operation with a large number of donors. The remnant liver of the RD group, which was smallest, regenerated fastest in the donor group. The donor remnant liver may not regenerate to the full volume that it had before the operation. However, most of the donors achieved normal liver synthetic function within 1 postoperative week and without complications. The difference in liver regeneration between donor and recipient was also comparable. The donor remnant liver seemed to regenerate slowly compared with the recipient grafted liver. An accurate report on liver regeneration as well as liver function helps to ensure proper informed consent. Donor hepatectomy, including right hepatectomy, can be safely performed with accurate preoperative volumetry and careful decision-making concerning graft-type selection because appropriate regeneration occurred and donors showed normal liver function.

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British Journal of Cancer (2008) 99, 1891 – 1899

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Loss of imprinting of *IGF2* correlates with hypermethylation of the *H19* differentially methylated region in hepatoblastoma

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IGF2, a maternally imprinted foetal growth factor gene, is implicated in many childhood tumours including hepatoblastoma (HB); however, the genetic and epigenetic alterations have not comprehensively been studied. We analysed the methylation status of the *H19* differentially methylated region (DMR), loss of heterozygosity (LOH) and allelic expression of *IGF2* in 54 *HB* tumours, and found that 12 tumours (22%) with LOH, 9 (17%) with loss of imprinting (LOI) and 33 (61%) with retention of imprinting (ROI). Biallelic and monoallelic *IGF2* expressions correlated with hypermethylation and normal methylation of *H19* DMR, respectively, in two tumours with LOI and seven tumours with ROI. Quantitative RT–PCR analysis showed minimal expression of *H19* mRNA and substantial expression of *IGF2* mRNA in tumours with LOH or LOI, and substantial expression of both *H19* and *IGF2* mRNAs in tumours with ROI. Increased *IGF2* expression with predominant embryonic P3 transcript was found in the majority of HBs with ROI and foetal livers. In contrast to the earlier reports, our findings suggest that the disruption of the enhancer competition model reported in Wilms' tumour may also occur in HB. Both frequencies of LOH and LOI seem to be lower in HB than in Wilms' tumour, reflecting the different tissue origins.

British Journal of Cancer (2008) **99,** 1891 – 1899. doi:10.1038/sj.bjc.6604754 www.bjcancer.com
Published online 28 October 2008

Keywords: hepatoblastoma; *IGF2*; *H19*; loss of heterozygosity; loss of imprinting

Hepatoblastoma (HB) is a rare malignant neoplasm of the liver, with an incidence of 0.5–1.5 per million children (Perilongo and Shafford, 1999). Remarkable progress in clinical outcome has been achieved in the past 20 years because of advances in chemotherapy and surgical procedures; however, the mortality rate remains 20–30% and treatment results in patients in advanced stages who are refractory to standard preoperative chemotherapy regimens are unsatisfactory (Perilongo *et al*, 2000; Fuchs *et al*, 2002). To improve the mortality of these patients, innovative treatment based on a specific molecular target is needed. The molecular mechanism involved in the development and progression of HB includes overexpression of insulin-like growth factor-II (*IGF2*) (Li *et al*, 1998b; Gray *et al*, 2000; Hartmann *et al*, 2000), downregulation of *RASSF1A* by promoter hypermethylation (Sugawara *et al*, 2007; Honda *et al*, 2008) and alterations of genes in the Wnt signalling pathway; most notably, the high incidence of *CTNNB1* (catenin, β1) mutation (Koch *et al*, 1999; Taniguchi *et al*, 2002).

IGF2 is a maternally imprinted gene and encodes a foetal peptide hormone that regulates cellular proliferation and differentiation (Foulstone *et al*, 2005). *IGF2* has four promoter regions and P3 is the most active promoter in the foetal liver, followed by P2 and P4 promoters (Li *et al*, 1998a). *PLAG1* encodes a developmentally regulated transcription factor, which positively regulates *IGF2* through binding the P3 promoter region. Although *IGF2* is downregulated in normal tissues after birth, except for liver tissues, it is overexpressed in a wide variety of childhood and adult cancers and serves as a tumour enhancer through autocrine and paracrine mechanisms (Toretsky and Helman, 1996). *IGF2* has been studied extensively over the past decade as a key molecule involving HB and Wilms' tumour (WT) pathogenesis.

The allelic expression of *IGF2* is regulated by the methylation status of the sixth CTCF (CCCTC-binding factor) site in the *H19* differentially methylated region (DMR) that represents the parental origin of the *IGF2* allele; whereas the paternal CTCF6 allele is methylated, the maternal allele is unmethylated in normal tissues (Bell and Felsenfeld, 2000; Hark *et al*, 2000; Takai *et al*, 2001). Using the enhancer competition model, *IGF2* and *H19* promoters compete on the same chromosome for a shared enhancer, and access of the maternal *IGF2* allele to this enhancer is blocked by *H19* DMR when unmethylated because of the insulator activity of CTCF binding to unmethylated *H19* DMR (Bell and Felsenfeld, 2000; Hark *et al*, 2000). It has been proved in many WTs that aberrant methylation of the maternal CTCF6 prevents the insulator binding and leads to loss of imprinting (LOI), resulting in the overexpression of *IGF2* (Steenman *et al*, 1994; Ravenel *et al*, 2001). Although LOI of *IGF2* was reported in HB, the mechanism of LOI, the concurrent overexpression of *IGF2* mRNA and loss of

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Received 24 June 2008; revised 30 September 2008; accepted 1 October 2008; published online 28 October 2008

H19 mRNA expression are uncertain because of the limited number of HB tumours examined and the low frequency of the heterozygous *IGF2* polymorphic site in general populations (Davies, 1993; Montagna *et al*, 1994; Rainier *et al*, 1995; Li *et al*, 1995, 1998b; Fukuzawa *et al*, 1999; Gray *et al*, 2000; Hartmann *et al*, 2000; Ross *et al*, 2000; Albrecht *et al*, 2004; Suzuki *et al*, 2008), and some investigators stated earlier that the mechanisms of *IGF2* upregulation by LOI found in WT do not apply to HB (Li *et al*, 1995; Hartmann *et al*, 2000).

Loss of imprinting was reported in 32–38% of WTs (Ravenel *et al*, 2001; Fukuzawa *et al*, 2004; Yuan *et al*, 2005), and loss of heterozygosity (LOH), leading to uniparental disomy (UPD) of the paternal *IGF2*, was reported in 36–50% of WTs (Grundy *et al*, 1996; Fukuzawa *et al*, 2004; Yuan *et al*, 2005). In HB, although LOH of *IGF2* was reported in 20–30%, the incidence of LOI of *IGF2* was uncertain because each series included only a small number of HB tumours. In addition, it is also uncertain whether the same mechanism of LOI is involved in both WT and HB tumorigeneses because the methylation status of *H19* DMR in HB has rarely been examined (Li *et al*, 1995, 1998b; Fukuzawa *et al*, 1999).

To determine whether the alterations of *IGF2* and *H19* loci identified in WT are also found in HB, we examined the LOI and LOH status of *IGF2* using combined bisulphite restriction assay (COBRA) of the CTCF6 region that can determine the methylation status of *H19* DMR more efficiently than the method using methylation-specific restriction enzymes and Southern blot in 54 HB tumours. In addition, we evaluated promoter-specific *IGF2* transcripts, the methylation status of *IGF2* promoters and *PLAG1* mRNA expression. Our results showed that the genetic and epigenetic alterations in the *IGF2-H19* region with elevated expression of *IGF2* mRNA identified in WTs were also found in the great majority of HB tumours, although the incidences of LOH and LOI may be lower in HBs than in WTs.

MATERIALS AND METHODS

Patients and samples

Tumour tissues were obtained from 54 Japanese children with HB, and adjacent normal liver tissues were available from 5 patients. Eighteen tumour and five matched normal liver specimens were supplied by the Tissue Bank of the Japanese Study Group for Pediatric Liver Tumour (JPLT) (Matsunaga *et al*, 2004), and 36 were supplied by institutions affiliated with Saitama Cancer Center. DNA and RNA were extracted from tumour and normal tissue samples that were immediately frozen after the resection or on arrival at the centre. The median age of the 54 patients at diagnosis was 18 months (range, 1–156 months). None of patients had the Beckwith–Wiedemann syndrome or a family history of familial polyposis coli. A total of 14 and 37 tumours were obtained before and after chemotherapy, respectively, and the chemotherapy status was unknown in the other 3 tumours. Pathologists in each institution and/or the JPLT pathology panel made the diagnosis of HB and verified that each sample contained 70% or more tumour cells. Informed consent was obtained from the parents, and the study design was approved by the ethics committee of Saitama Cancer Center.

COBRA of the CTCF6 site at *H19* DMR

We performed COBRA to determine the methylation status of the CTCF6 binding site at *H19* DMR, as described earlier (Watanabe *et al*, 2007). COBRA of CTCF6 showed that the mean methylation percentage ±2 s.d. of five normal livers was 52.8 ± 15.0%, and we defined more than the mean percentage +2 s.d. as the hypermethylated state.

LOH analysis of *IGF2*

High-resolution single nucleotide polymorphism (SNP) array, Affymetrix Mapping 50K-Xba array (Affymetrix, Santa Clara, CA, USA), was used to analyse chromosomal aberrations of 11p15.5 where *IGF2* resides. Genomic DNA in 43 of 54 tumours and 2 cell lines was assayed according to the manufacturer's protocol, and the genomic status of *IGF2* was determined as described earlier (Haruta *et al*, 2008).

Allelic expression analysis of *IGF2* and quantitative real-time reverse transcription-PCR analysis of *IGF2* and *H19* mRNA

The *ApaI/AvaII* polymorphic site in exon 9 of *IGF2* was used to evaluate the allelic expression of *IGF2* mRNA in 21 tumours whose RNA was available for this study, as described earlier (Watanabe *et al*, 2006). Quantitative real-time reverse transcription-PCR was performed to evaluate the total *IGF2* and *H19* mRNA levels in 20 tumour tissues, 2 HB cell lines (HuH6 and HepG2), foetal liver total RNA pooled from 34 foetuses (Clontech, Ohtsu, Japan) and 3 normal liver tissues adjacent to HB; the age of the patients was 16, 24 or 26 months. Of the 20 tumours, 3 and 16 were obtained before and after chemotherapy, respectively, and the chemotherapy status was unknown in 1. The primers and TaqMan probes used for *IGF2* and *H19* mRNA were described earlier (Watanabe *et al*, 2007; Haruta *et al*, 2008). The expression of *IGF2* and *H19* mRNAs was normalised with *GAPDH*.

Methylation-specific PCR and bisulphite sequencing analysis of *IGF2* promoter regions

Genomic DNA from tumour and normal liver samples was treated with sodium bisulphite (Herman *et al*, 1996), and the methylation status of the P2–P4 promoter regions of *IGF2* was analysed by methylation-specific PCR (MSP), as described earlier (Beeghly *et al*, 2007). Polymerase chain reaction products were run on 2% agarose gels and visualised after staining with ethidium bromide. We confirmed the results of MSP analysis of P3 promoter by bisulphite sequencing of eight or more subcloned plasmids.

Semiquantitative RT–PCR analysis of promoter-specific transcripts of *IGF2* and *PLAG1*

P1 and P3 promoter specific expressions of *IGF2* mRNA were analysed using the primer sets described elsewhere (Lu *et al*, 2006). The primer sequences for P2-specific transcript were derived from exons 4 and 5: forward, 5′-CCCTCAGGACGTGGACAG-3′; reverse, 5′-GTGCGTTGGACTTGCATAGA-3′; and the primer sequences for P4-specific transcript were derived from exons 7, 8 and 9: forward, 5′-CGAGCCTTCTGCTGAGCTAC-3′; reverse, 5′-CGGAAACAGCA CTCCTCAAC-3′. *PLAG1* mRNA expression was analysed using the following primer sets: forward, 5′-AACGTAAGCGTGGTGAAACC-3′; reverse, 5′-TGCCACATTCTTCGCACTTA-3′ (Zatkova *et al*, 2004). Polymerase chain reaction products were run on polyacrylamide gels and visualised after ethidium bromide staining. The intensity of each band was examined using a fluorescence image analyser, FLA-3000G (Fujifilm, Tokyo, Japan). Dividing the intensity of the target transcript by that of *GAPDH* calculated the level of each transcript.

Mutation analysis of the *CTNNB1* gene

To detect point mutations and deletions of the *CTNNB1* gene, genomic DNA from each tumour sample was amplified using two sets of primers, F1, 5′-TGGCTATCATTCTGCTTTTCTTG-3′ and R1, 5′-CTCTTTTCTTCACCACAACATTTT-3′, and BCAT-3, 5′-AA

AATCCAGCGTGGACAATGG-3′ and BCAT-4, 5′-TGTGGCAAGTT CTGCATCATC-3′, respectively (Koch *et al*, 1999; Satoh *et al*, 2003). The PCR products were either directly sequenced or inserted into a vector (pGEM (R)-T Easy Vector System (Promega, Madison, WI, USA)), and six or more clones were sequenced.

Statistical analysis

Student's *t*-test or Welch's *t*-test compared mRNA levels of *IGF2* and *H19* between tumours with or without *IGF2* alterations or other characteristics and the levels of *IGF2* promoter-specific transcripts between tumours with or without *PLAG1* mRNA expression. We also assessed the association between total *IGF2* mRNA levels and P2-, P3- or P4-specific *IGF2* mRNA levels by determining the Spearman rank correlation coefficient and associated *P*-value. Differences in the incidence of tumours with unmethylated P3 promoter were examined between tumours with hypermethylated *H19* DMR and tumours with normally methylated *H19* DMR by the χ^2 test. Differences in the incidences of tumours with *CTNNB1* mutation were examined between any two of three groups of tumours classified on the basis of the *IGF2* status by the χ^2 test.

RESULTS

Methylation status of the CTCF6 binding site at *H19* DMR, LOH analysis using SNP array and allelic expression analysis of *IGF2*

Combined bisulphite restriction assay showed that 21 and 33 tumours had hypermethylation and normal methylation at CTCF6, indicating LOH or LOI and retention of *IGF2* imprinting (ROI), respectively (Table 1 and Figure 1). Single nucleotide polymorphism array analysis was performed in 43 of 54 tumours; all 21 tumours with hypermethylated CTCF6 and 22 of 33 tumours with normally methylated CTCF6. Combined results of both analyses indicated that 12 tumours had LOH (10 hypermethylated CTCF6 and UPD 2 hypermethylated CTCF6 and hemizygous 11p15 deletion), 9 had LOI (hypermethylated CTCF6 and retention of heterozygosity (ROH)) and 22 had ROI (normally methylated CTCF6 and ROH). Of 21 tumours whose RNA was available, 9 and 12 tumours had heterozygous and homozygous *ApaI*/*Ava*II sites in exon 9 of *IGF2*, respectively. Of the nine heterozygous tumours, seven showed monoallelic expression of *IGF2*, indicating ROI, and two showed biallelic expression of *IGF2*, indicating LOI, and the results were consistent with those examined by COBRA and SNP array analyses (Table 1). From these findings, 11 tumours with normally methylated CTCF6, in which SNP array analysis was not performed, were classified as those with ROI. Thus, combined results of COBRA, SNP array and allelic expression analyses showed 12 tumours with LOH, 9 tumours with LOI and 33 tumours with ROI. In addition, one cell line (HuH6) had LOI, and the other (HepG2) had LOH (UPD) of *IGF2*.

The mean age was compared between any two of three groups of patients (i.e., LOH, LOI or ROI) by Student's *t*-test. There was no difference in the mean age between any two of the three groups of patients.

Correlation between *IGF2* and *H19* mRNA levels and the *IGF2* status (LOH, LOI or ROI)

Quantitative real-time reverse transcription-PCR analysis showed that although 15 of 20 tumours had a higher level of *IGF2* mRNA than normal liver tissues, 15 of 20 tumours had a lower level of *H19* mRNA than normal liver tissues (Table 1 and Figure 2). All 3 tumours with UPD, 1 of 1 with 11p15 loss, 1 of 3 with LOI and 10 of 13 with ROI, expressed higher levels of *IGF2* mRNA than normal liver tissues. There was no significant difference in *IGF2* mRNA levels between 3 tumours with UPD or 7 tumours with *IGF2* alterations; that is UPD, 11p15 loss or LOI, and 13 tumours with ROI. In contrast, 7 tumours with *IGF2* alterations expressed very low levels of *H19* mRNA, whereas 11 of 13 tumours with ROI expressed a substantial amount of *H19* mRNA; 2 tumours (nos. 25 and 27) with ROI expressed very low levels of *H19* mRNA. *H19* mRNA levels were higher in 13 HB tumours with ROI than in 7 HB tumours with *IGF2* alterations ($P<0.01$ by Welch's *t*-test). Although HepG2 with UPD had a higher level of *IGF2* mRNA than normal liver tissues, HuH6 with LOI had a very low level of *IGF2* mRNA. *H19* mRNA levels were very low in both cell lines.

Semiquantitative RT–PCR analysis of promoter-specific *IGF2* transcripts

Because the *IGF2* gene has four kinds of promoters, promoter-specific *IGF2* transcripts were analysed to determine the usage of each promoter. Representative results of the P3 transcript are shown in Figure 3A. All 20 tumours showed undetectable or lower levels of P1 transcripts than 3 normal liver tissues. The levels of P2, P3 and P4 transcripts were higher in 13, 15 and 10 of the 20 tumours, respectively, than those of normal liver tissues. Polymerase chain reaction cycle numbers to obtain visible levels of PCR products were 40 for P2 transcripts, 30 for P3 transcripts and 35 for P4 transcripts, indicating that the amounts of P3 transcripts were high, those of P2 transcripts were low and those of P4 transcripts were intermediate. The Spearman correlation coefficient analysis showed that the expression levels of the P2, P3 and P4 transcripts correlated with the levels of total *IGF2* mRNA (P2, $rS=0.730$; P3, $rS=0.773$ and P4, $rS=0.646$) (Figure 3B and C; data for the P2 and P4 transcripts are not shown).

The methylation status of *IGF2* promoters and its correlation with the levels of promoter-specific transcripts

In the MSP analysis of each promoter, the P2 promoter region was partially methylated in 19 tumours and normal liver tissues and the P4 promoter region was unmethylated in all 20 tumours and normal liver tissues. Therefore, the methylation status of P2 or P4 promoter region was not correlated with the expression level of P2- or P4-specific transcripts. The P3 promoter region was partially methylated in 11 tumours, HuH6 and normal liver tissues and unmethylated in 9 tumours and HepG2 (Table 1, Figure 4A and B). The results of MSP analysis in one tumour (no. 1) and HuH6 were confirmed by bisulphite sequencing (Figure 4C). Nine tumours with the unmethylated P3 promoter had higher levels of P3 transcripts than 11 tumours with the partially methylated P3 promoter ($P=0.005$) (Figure 4D). The P3 promoter was unmethylated in 5 of 7 tumours with *IGF2* alterations; UPD, 11p15 loss or LOI, but in 4 of 13 tumours with ROI. Thus, the incidence of tumours with unmethylated P3 promoter tended to be higher in tumours with hypermethylated *H19* DMR than in tumours with normally methylated *H19* DMR ($P=0.1$).

Semiquantitative RT–PCR analysis of *PLAG1* mRNA

PLAG1 positively regulates *IGF2*, and its expression was detected in 12 tumours, foetal liver RNA and 2 cell lines, but not in 8 tumour and 3 normal liver tissues (Table 1 and Figure 5). The 12 tumours with *PLAG1* mRNA expression showed higher levels of P4-specific *IGF2* transcripts ($P=0.01$) and tended to show higher levels of P3-specific *IGF2* transcripts ($P=0.051$) than the 8 tumours without *PLAG1* expression. There was no significant difference in P2- or P1-specific transcript levels between tumours with and without *PLAG1* mRNA expression.

Table 1 Genetic and epigenetic status of the *IGF2-H19* region in 54 hepatoblastoma tumours

Patients number	Age[a]/sex	Chemo[b]	%methyl CTCF6[c]	11p15 SNP[d]	*ApaI* site[e]	*IGF2* RT-PCR	*IGF2* status[f]	*IGF2* mRNA	P1E[g]	P2M[h]	P2E	P3M	P3E	P4E	*H19* mRNA	*PLAG1* mRNA	*CTNNB1* status[i]
1	48/F	+	82.8	UPD	Homo	ND	UPD	11.3	0.6	MU	17.7	U	7	3.1	0	+	M
2	5/M	–	93.4	UPD	Homo	ND	UPD	3.9	0	MU	0	U	2.1	0.9	0	–	M
3	24/M	+	76.7	UPD	Homo	ND	UPD	3.2	0	MU	2.4	MU	2.3	1.2	0	+	M
4–10	5–96/M6, F1	+6, –1	72–90	UPD	ND	ND	UPD	ND	ND	ND	ND	ND	ND	ND	ND	ND	M4, N3
11	27/M	+	87.8	Loss chr II	Homo	ND	Loss	6.1	0	MU	2.3	U	4.4	1.3	0	+	M
12	24/M	+	81.9	Loss chr II	ND	ND	Loss	ND	ND	ND	ND	ND	ND	ND	ND	ND	M
13	12/F	+	91.4	ROH	Homo	ND	LOI (m)	9.7	0	U	1.1	U	2.3	0.6	0	–	ND
14	16/M	–	86.1	ROH	Homo	ND	LOI (m)	1	0	MU	0.3	U	1.3	0.9	0	–	ND
15	26/F	+	83.1	ROH	Hetero	LOI	LOI (m, p)	0.8	0	MU	0.4	MU	0.6	0.9	0	–	M
16	24/M	+	70.9	ROH	Hetero	LOI	LOI (m, p)	ND	ND	ND	ND	ND	ND	ND	ND	ND	M
17–21	12–84/M4, F1	+1, –3, UK1	71–91	ROH	ND	ND	LOI (m)	ND	ND	ND	ND	ND	ND	ND	ND	ND	M3,N2
22	12/F	+	52.5	ND	Homo	ND	ROI (m)	9.2	0.8	MU	8.4	MU	2.8	2.2	3.5	+	N
23	109/F	+	49.1	ND	Homo	ND	ROI (m)	8.4	0	MU	4.3	U	4.6	2.8	0.5	+	M
24	12/M	–	56.3	ND	Hetero	ROI	ROI (m, p)	7.4	0	MU	13.2	U	4.6	2.4	1.9	+	M
25	15/M	+	55.9	ROH	Hetero	ROI	ROI (m, p)	5.7	0	MU	6.5	MU	5.1	2.2	0	+	ND
26	6/M	UK	51.1	ND	Hetero	ROI	ROI (m, p)	5.6	0.1	MU	4.7	U	1.2	0.5	2.7	+	ND
27	10/M	+	62.1	ROH	Homo	ND	ROI (m)	5	0	MU	0.9	U	5	1.1	0	–	ND
28	29/F	+	61.5	ND	Homo	ND	ROI (m)	3.7	0	MU	1.5	MU	2.5	1.4	2.5	+	M
29	26/M	+	55.4	ND	Homo	ND	ROI (m)	3.1	0.2	MU	9.3	MU	0.4	1.2	0.7	–	M
30	18/M	+	48.7	ND	Hetero	ROI	ROI (m, p)	2.4	0.1	MU	1.2	MU	1.1	1	0.8	–	ND
31	13/M	+	55.3	ND	Hetero	ROI	ROI (m, p)	2.2	0	MU	1.1	MU	1.5	1	0.6	+	N
32	60/F	+	55.7	ND	Hetero	ROI	ROI (m, p)	0.7	0.2	MU	0.2	MU	0.3	0.9	0.4	+	N
33	29/M	+	56.4	ND	Homo	ND	ROI (m)	0.5	1	MU	0.1	MU	0	0.5	1.2	–	M
34	9/M	+	56.7	ND	Hetero	ROI	ROI (m, p)	0.5	0	MU	0	MU	0.1	0.2	0.2	+	M
35–54	4–156/M11, F9	+12, –7, UK1	41–65	ROH	ND	ND	ROI (m)	ND	ND	ND	ND	ND	ND	ND	ND	ND	M11, N9
Normal livers			52.8	ND	ND	ND	ROI (m)	1	1	MU	1	MU	1	1	1	–	
Fetal livers			ND		ND	ND	ND	5.9	0.2	ND	6.4	ND	4.8	1.9	2.4	ND	
HuH6			87.3	ROH	Hetero	LOI	LOI (m, p)	0	0	MU	0	MU	0	0.1	0	+	M
HepG2			89.5	UPD	Homo	ND	UPD (m)	2.4	0	U	1.5	U	5	2.8	0	+	M

F = female; M = male; M = methylated; ROH = retention of heterozygosity; ROI = retention of imprinting; U = unmethylated; ND = not done; UK = unknown. All 20 tumours showed unmethylated promoter 4; UPD, uniparental disomy; loss chr 11, loss of chromosome 11or 11p15; LOI, loss of imprinting. [a]Age in months. [b]Chemo, chemotherapy before surgery; +6, –1 indicates that six and one tumours were treated and untreated, respectively, with chemotherapy before surgery. [c]%methyl CTCF6 indicates % methylated CTCF6 allele. [d]Results of SNP array. [e]Homo, homozygosity at *ApaI*/*AvaII* site; hetero, heterozygosity. [f]Results of SNP array analysis, methylation analysis of CTCF6 (m) and *ApaI*/*AvaII* polymorphism site analysis (p). [g]P1E, promoter 1-specific transcript. [h]P2M, the methylation status of promoter 2. [i]*CTNNB1* status: M, mutated; N, normal.

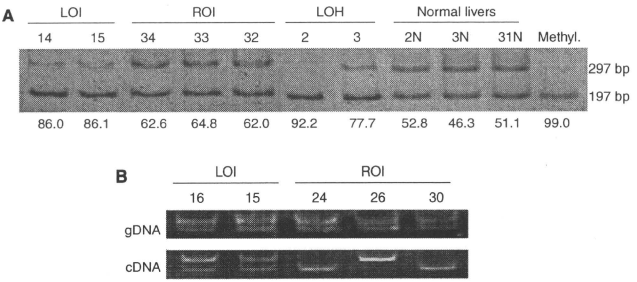

Figure 1 Analysis of *IGF2* alterations. (**A**) Examples of the methylation status of CTCF6 analysed by a combined bisulphite restriction assay (COBRA). Bisulphite-modified PCR products were digested with *MluI*. Upper and lower lanes indicate unmethylated and methylated fragments, respectively. Numbers above lanes indicate the tumour number. Numbers below lanes show the percentage of methylated DNA fragments containing CTCF6. The mean value of the DNA methylation percentages calculated from three COBRA experiments is shown in Table 1. Methyl., control methylated DNA. The *IGF2* status is shown above the tumour numbers. LOI, loss of *IGF2* imprinting; LOH, loss of heterozygosity in the *IGF2* region; ROI, retention of *IGF2* imprinting. (**B**) Electrophoretic pattern of genomic DNA PCR products or RT–PCR products after *AvaII* digestion. Reverse transcriptase–PCR analysis shows LOI in two tumours and ROI in three tumours.

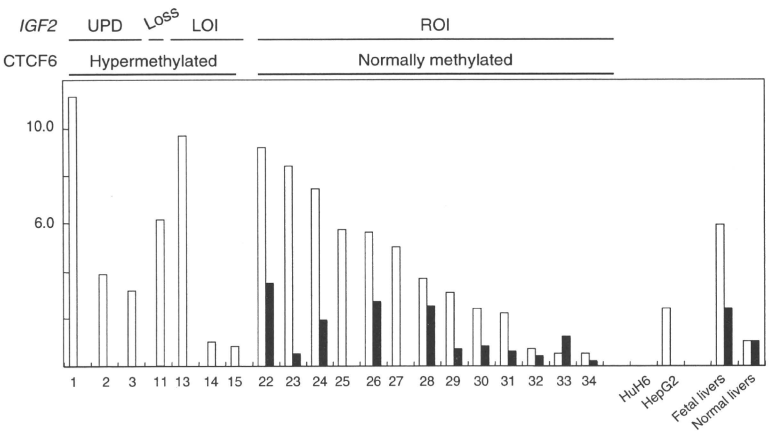

Figure 2 Results of quantitative real-time RT–PCR analysis of *IGF2* and *H19* mRNAs. Relative mRNA (Y axis) of total *IGF2* (open rectangles) and *H19* (closed rectangles) is plotted in 3 tumours with UPD, in 1 tumour with 11p15 loss, in 3 tumours with LOI, in 13 tumours with ROI, in 2 cell lines, in foetal liver total RNA and in adjacent normal liver tissues (a mean value of 3 samples). Tumours in each group are arranged in order by the levels of *IGF2* mRNA. Numbers below X axis indicate the tumour number shown in Table 1. *IGF2* status (UPD, loss of 11p15, LOI and ROI) and methylation status of CTCF6 at *H19* DMR (hypermethylated or normally methylated) are shown above the graph. Nine tumours (nos. 1–3, 11, 13–15, 25 and 27) and two cell lines expressed a minimal amount of *H19* mRNA, which was shown as zero in the graph. Similarly, HuH6 expressed a minimal amount of *IGF2* mRNA, which was shown as zero in the graph.

Incidences of tumours with *CTNNB1* mutation between any two groups of tumours classified on the basis of the *IGF2* status

DNA was available for *CTNNB1* mutation analysis in 48 of 54 HB tumours. The results are described in Table 1. There were no differences in the incidences of *CTNNB1* mutation between 7 tumours with *IGF2*-LOI and 29 tumours with *IGF2*-ROI or 12 tumours with *IGF2*-LOH, and between 29 tumours with *IGF2*-ROI and 12 tumours with *IGF2*-LOH.

DISCUSSION

In this study, biallelic and monoallelic *IGF2* expressions correlated with hypermethylation and normal methylation of CTCF6,

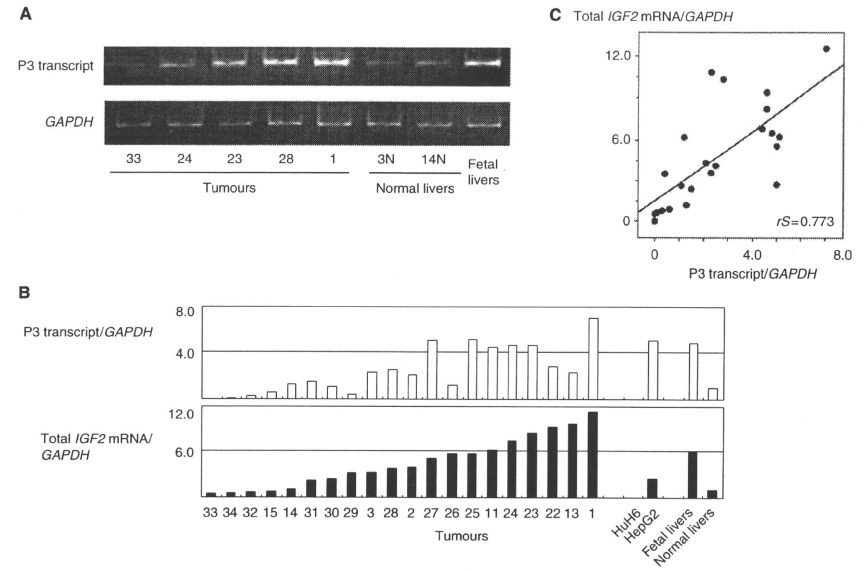

Figure 3 (**A**) Representative data of RT–PCR analysis of P3 transcripts. (**B**) Expression levels of P3 transcripts (upper lane) and total *IGF2* mRNA (lower lane) are plotted in 20 tumours, in 2 cell lines, in foetal liver tissues and normal liver tissues (a mean value of 3 samples). Tumours are arranged in order by total levels of *IGF2* mRNA. Numbers below *X* axis indicate the tumour number. (**C**) Correlation between levels of P3 transcript (*X* axis) and total *IGF2* mRNA (*Y* axis).

respectively, in two tumours with LOI and seven tumours with ROI (Table 1, Figure 1). In addition, the paternal origin of the duplicated *IGF2* loci was confirmed by the hypermethylated CTCF6 in 10 tumours with UPD. Furthermore, very low expression levels of *H19* mRNAs and substantial expression levels of *IGF2* mRNAs in HB tumours with UPD or LOI, and substantial expression levels of both *IGF2* and *H19* mRNA in HB tumours with ROI were found (Table 1 and Figure 2). Two (nos. 14 and 15) of three HB tumours with LOI expressed *IGF2* mRNA levels comparable to but not higher than those of *IGF2* mRNA in normal liver tissues. In addition, one cell line, HuH6, with LOI expressed minimal expression of *IGF2* mRNA, although Hartmann *et al* (2000) found the moderate expression in the same cell line. These findings may be explained by the speculation that such tumours expressed increased levels of *IGF2* mRNA at the critical time of tumorigenesis, but not at the time of surgical resection or after many passages of cell culture. From these findings, the hypothesis established for WT that the hypermethylation of maternal *H19* DMR causes LOI, and that LOI or duplication of paternal *IGF2* (UPD) results in overexpression of *IGF2*, may be also applied to HB.

Although the expression levels of *IGF2* mRNA were reported to be higher in WTs with UPD than in WTs with ROI in two series of WTs (Wang *et al*, 1996; Haruta *et al*, 2008), conflicting results were reported in *IGF2* mRNA levels between WTs with LOI and WTs with ROI (Wang *et al*, 1996; Ravenel *et al*, 2001). The present and earlier studies showed that all HB tumours with UPD and the majority of HB tumours with LOI or ROI expressed the higher levels of *IGF2* mRNA than normal liver tissues (Li *et al*, 1998b; Gray *et al*, 2000; Hartmann *et al*, 2000). This study also showed that P3 transcripts predominated in total *IGF2* mRNAs in HB tumours irrespective of the *IGF2* status (i.e., UPD, 11p15 loss, LOI or ROI); these findings were similar to those reported in foetal liver tissues showing elevated expression of *IGF2* mRNA with predominance of the P3 transcript (Li *et al*, 1998a). Thus, the high *IGF2* mRNA expression of many HB tumours with ROI may mimic the upregulation of *IGF2* expression in embryonic liver tissues, from which HB may arise.

In this study of 54 HB tumours, we found LOH in 12 (22.2%), LOI in 9 (16.7%) and ROI in 33 (61.1%). Hepatoblastoma tumours can be classified into those with LOH and those with ROH, and tumours with ROH can be further classified into those with LOI and those with ROI. For data comparison, the frequencies of LOH and LOI in the earlier and present series of HB tumours are shown in Tables 2 and 3, respectively (Davies, 1993; Montagna *et al*, 1994; Li *et al*, 1995; Rainier *et al*, 1995; Fukuzawa *et al*, 1999; Gray *et al*, 2000; Hartmann *et al*, 2000; Ross *et al*, 2000; Albrecht *et al*, 2004; Suzuki *et al*, 2008). Both frequencies of LOH and LOI were similar between the earlier and present series of HB tumours. When we compared the frequencies of LOH and LOI between HB and WT, the frequencies of LOH and LOI are lower in HB tumours than in WT tumours (Table 4). The present and earlier studies showed that levels of *IGF2* mRNA are higher in normal liver tissues than in normal kidney tissues, and in foetal liver tissues than in foetal kidney tissues, but showed similarly high levels in both WTs and HBs (part of the data not shown) (Hedborg *et al*, 1994; Haruta