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H. 知的財産権の出願・登録状況

緒方勤. Estrogen receptor alpha gene, genomic DNA, and diagnosis marker. 米

国特許出願 Patent No: US 7,601,828

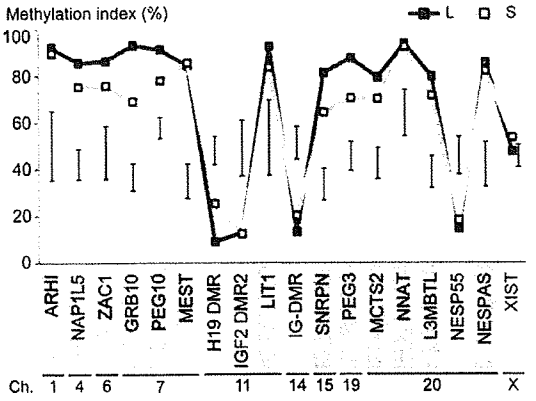

図1. ゲノム上全DMAのスクリーニングシステムの開発。世界初の全染色体母親性ダイソミー

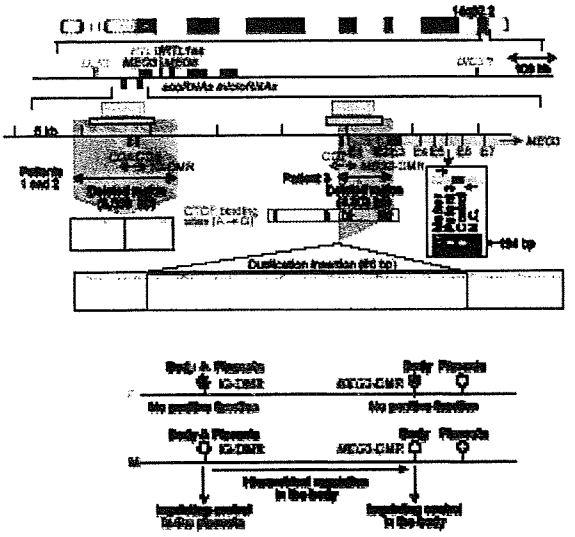

図2. 第14染色体インプリンティング領域の構造とIG-DMRあるいはMEG3-DMRのみを欠失した患者における欠失領域、および、IG-DMRとMEG3-DMRの機能を示すシェーマ。

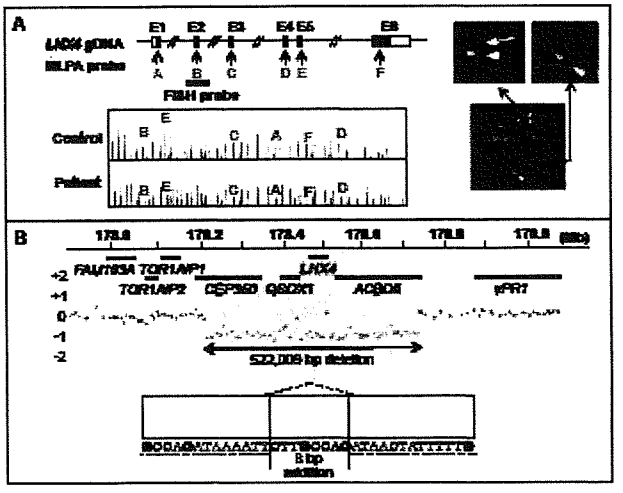

図3. MLPA法によるLHX4遺伝子の欠失。kの欠失はFISHおよびアリゴアレイCGHで確認され、欠失範囲も確定している。

研究成果の刊行一覧表

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研究成果の刊行物・別刷り

Pediatr Surg Int
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ORIGINAL ARTICLE

Outcome of pediatric renal tumor treated using the Japan Wilms Tumor Study-1 (JWiTS-1) protocol: a report from the JWiTS Group

Takaharu Oue · Masahiro Fukuzawa · Hajime Okita · Hideo Mugishima · Hiroshi Horie · Jun-ichi Hata · Masahiro Saito · Miwako Nozaki · Motoaki Chin · Hisaya Nakadate · Shiro Hinotsu · Tsugumichi Koshinaga · Yasuhiko Kaneko · Yoshihiro Kitano · Yukichi Tanaka · Japan Wilms Tumor Study (JWiTS) Group

Abstract

Purpose In 1996, the Japan Wilms Tumor Study (JWiTS) group was founded to elucidate the efficacy and safety of the regimen established by the National Wilms Tumor Study (NWTS) group in the USA, and a multicenter cooperative study (JWiTS-1) was started in Japan. This report reviews the results of JWiTS-1.

Methods A total of 307 patients with malignant renal tumor were enrolled in the JWiTS-1 study between 1996 and 2005. Central pathological diagnosis and follow-up data were available in 210 cases. The protocol regimens were similar to the NWTS-5 regimens. Clinical stage was classified according to the Japanese Staging System.

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Results Five-year overall survival (OS) rate was 91.1% for nephroblastoma, 72.9% for clear cell sarcoma of the kidney (CCSK), and 22.2% for rhabdoid tumor of the kidney (RTK). In the nephroblastoma patients, 5-year OS was 90.5% for stage I disease, 92.2% for stage II, 90.9% for stage III, 86.7% for stage IV, and 78.7% for stage V.
Conclusions The OS of patients in the JWiTS-1 study were comparable with the results of other multicenter studies in the USA and Europe. The outcome for patients with nephroblastoma and CCSK was fair. In contrast, the cure rate for those with RTK was not satisfactory. New treatment strategies are needed for patients with RTK.

Keywords Clinical trials · Group study · Japan · Survival · Wilms tumor

Introduction

Wilms tumor (WT), or nephroblastoma, is the most common malignant renal tumor in childhood. The survival of patients with WT has improved dramatically from 30% just a few decades ago to almost 90% in the modern era [1]. The improvements in survival have occurred as the result of advances in multimodality treatments including surgical management, irradiation, and chemotherapy, established in trials and studies conducted by many national and international cooperative groups. In the USA and Canada, the National Wilms Tumor Study (NWTS) group, now part of the Children's Oncology Group (COG), has studied the therapy and outcomes of children with WT since 1969 [2]. In Europe, international cooperative studies have been conducted predominantly by the International Society of Paediatric Oncology (SIOP) since 1971 [3].

The goals of these groups are to increase cure rates while minimizing morbidity. In NWTS studies primary surgical resection of the tumor was the initial treatment of most children, whereas in SIOP studies chemotherapy was the initial treatment. Both approaches have distinct advantages and disadvantages. The benefit of the NWTS approach is that it enables accurate assessment of histology, extent, and molecular biology of the untreated tumor. However, the resection of large tumors sometimes results in intraoperative tumor spillage, which increases the risk of local abdominal relapse and subsequent poor outcome [4]. On the other hand, the benefit of the SIOP approach is that preoperative chemotherapy usually reduces tumor volume, thereby decreasing the likelihood of spillage and downstaging the tumor [5]. Moreover, clinical and histological responses to the chemotherapy may provide valuable prognostic information [6]. Most patients treated on SIOP WT studies do not undergo tumor biopsy before starting chemotherapy. Therefore, patients who are not subsequently diagnosed as having WT may have received unnecessary therapy. In the SIOP 93-01 trial approximately 5% of lesions in patients treated with chemotherapy were ultimately shown not to be WT and 1.8% were benign [7]. Moreover, the true extent of disease may be masked by pretreatment.

Before 1996, Japanese children with renal tumor were treated individually at local institutions or by doctors using protocols developed by the NWTS. However, exact incidence and prognosis were unclear, and the survival rates of patients with stages III and IV WT were 10–15% worse than those in Western countries. The 5-year survival rate of children with WT registered with the Japanese Society of Pediatric Surgeons between 1991 and 1995 was 81.8% in stage III and 57.1% in stage IV disease, whereas the 4-year survival rate of patients registered to the NWTS-4 trial (1986–1994) was 90.9% in cases of stage III disease and 80.9% in stage IV [8, 9]. To improve the outcome of children with renal tumor the Japan Wilms Tumor Study (JWiTS) group was founded in 1996, and a nationwide multicenter cooperative study (JWiTS-1) was started. At that time, the Japanese Staging System defined by the Japanese Society of Pediatric Surgeons was broadly used in Japan. The patients enrolled into JWiTS-1 study were classified using the Japanese Staging System to compare the prognosis between the patients treated before and after the introduction of JWiTS-1 study.

The objectives of the JWiTS were (1) to elucidate the efficacy and safety for Japanese patients of the regimen established by the NWTS group; (2) to start the multicenter cooperative study in Japan; (3) to establish a central system of pathological diagnosis; (4) to create a database of children with renal tumors; (5) to create a tissue bank for basic research to study the biology of renal tumors in childhood; and, finally, to improve the prognosis and quality of life of Japanese children with renal tumors. Here, we report the results of the JWiTS-1 study for pediatric renal tumor.

Materials and methods

Registration

JWiTS-1 was a multiinstitutional cooperative study for patients aged under 16 at diagnosis with primary untreated renal neoplasms. The study was approved by the ethics board of each local hospital, and informed consent was obtained from the parents before registration. Between 1996 and 2005, 307 patients with renal tumor were registered from 116 pediatric institutions. Among them, histological slides were submitted for central pathology in 269 cases (87.6%), and follow-up data were submitted in 229 cases (74.6%). Both follow-up data and central pathological

diagnosis were available in 210 cases (68.4%). Survival data were calculated in these cases.

Pathological diagnosis and clinical staging

Microscope slides, institutional pathology reports, and JWiTS pathology forms were sent and reviewed by JWiTS pathologists (J. Hata, H. Horie). The histology of each tumor was categorized as favorable or anaplastic WT, clear cell sarcoma of the kidney (CCSK), rhabdoid tumor of the kidney (RTK), or other tumor type, and a written report of the central review was sent to the patient's physician at the relevant institution.

Clinical stage was classified according to the Japanese Staging System defined by the Japanese Society of Pediatric Surgeons as follows: stage I, tumor limited to kidney, capsule is intact; stage II, tumor extends around the kidney (capsule, attached lymph nodes, renal vein, or pelvis); stage III, tumor extends to the surrounding organs such as aortic lymph nodes, ureter, or bladder; stage IV, hematogenous metastases are present; and stage V, bilateral renal tumor.

Treatments

The therapeutic strategy was similar to that of the NWTS-5 protocol. As a basic principle, all patients underwent an initial nephrectomy. Preoperative chemotherapy was performed only when the tumor seemed unresectable. The surgical procedure was performed via a transabdominal incision. Biopsies were performed on any lesion suspected of being WT. Both histological slides and snap-frozen tumor specimens were sent to the Central Pathologists and Tissue Preservation Center, respectively.

Patients received postoperative chemotherapy with or without radiation therapy according to pathological diagnosis and clinical stage defined by the Japanese Staging System as shown in Table 1. Chemotherapy regimens are listed in Table 2. The NWTS Group permitted to use these regimens. Patients with one or more pulmonary nodules identified on plain chest radiographs received 12 Gy to both lungs and the mediastinum. Patients under 2 years with stage I favorable WT, and whose tumor weighted <550 g, received no postoperative chemotherapy. However, shortly

Table 1 Treatment strategies used in the JWiTS-1 trial

Stage, histology	Radiation (cGy)	Chemotherapy regimen	Duration (weeks)
Stages I or II FH	None	EE-4A[a]	18
Stage I focal or diffuse anaplasia	None	EE-4A	18
Stages III or IV FH or stages II–IV focal anaplasia	1,080	DD-4A	24
Stages II–IV diffuse anaplasia, or CCSK	1,080	I	24
Stages I or II RTK	None	RTK	24
Stages III or IV RTK	1,080	RTK	24

CCSK clear cell sarcoma of the kidney, *FH* nephroblastoma with favorable histology, *RTK* rhabdoid tumor of the kidney

[a] Patients under 2 years with stage I favorable WT, and whose tumor weighed less than 550 g, received no postoperative chemotherapy until January 2003

Table 2 Treatment regimens used in the JWiTS-1 study

Regimen	Weeks																							
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
EE-4A	A			A			A			A			A			A			A						
		V	V	V	V	V	V	V	V	V	V		V*			V*			V*						
DD-4A	A			D			A			D			A			D*			A			D*			A
		V	V	V	V	V	V	V	V	V	V		V*			V*			V*			V*			V*
I	D			C			D			C			D			C			D			C			D
		V	V	E	V	V	V	V	V	E	V	V	V*	V*		E			V*			E			V*
							C*						C*						C*						C*
RTK	P			P			C			P			P			C			P			P			C
	E*			E*						E*			E*						E*			E*			

These regimens are same as the regimens used in NWTS-5. NWTS Group permitted to use these regimens

A actinomycin D (0.045 mg/kg, max 2.3 mg), *C* cyclophosphamide (14.7 mg/kg/day × 5), *C** cyclophosphamide (14.7 mg/kg/day × 3), *D* doxorubicin (1.5 mg/kg), *D** doxorubicin (1.0 mg/kg), *E* etoposide (3.3 mg/kg/day × 5), *E** etoposide (100 mg/m^2/day × 3), *P* carboplatinum (16.7 mg/kg/day × 2), *V* vincristine (0.05 mg/kg, max 2 mg), *V** vincristine (0.067 mg/kg, max 2 mg)

after the initiation of the JWiTS-1 trial recurrence was reported in these patients. These observations demonstrated that the postoperative chemotherapy is necessary even in patients with localized tumor. Therefore, in January 2003, the protocol was changed and postoperative chemotherapy was performed in all patients.

Statistical analysis

Survival time was defined as the time from diagnosis until death or last contact. Death regardless of cause and relapse were considered as an event. Overall survival (OS) and relapse-free survival (RFS) rates were calculated by the Kaplan–Meier method. Comparisons of the prognostic impact of each factor were performed using the log-rank test.

Results

Pathological diagnosis

Pathological review was completed for 87.6% (269 out of 307) of patients enrolled. According to the central pathological reports, 172 (75.1%) of the cases were nephroblastoma, 21 (9.2%) were RTK, 16 (7.0%) were CCSK, and 20 (8.7%) were other tumors such as congenital mesoblastic nephroma, renal sarcoma, and renal cell carcinoma. Anaplasia was observed in six out of 172 (3.5%) cases with nephroblastoma. Institutional and central pathological diagnoses were obtained in 197 cases, and the institutional diagnosis matched the central one in 161 (81.7%) of those cases. Table 3 shows some examples of the mismatching of institutional and central pathological diagnoses. Most cases were a misdiagnosis between favorable and unfavorable histology.

Table 3 Examples of the mismatching of institutional and central pathological diagnoses

Local diagnosis	Central diagnosis	Case no.
Nephroblastoma	CCSK	5
Nephroblastoma	RTK	1
Nephroblastoma	Nephroblastoma with anaplasia	3
Nephroblastoma with anaplasia	RTK	1
Nephroblastoma with anaplasia	Nephroblastoma, no anaplasia	7
CCSK	CMN	2
CCSK	Nephroblastoma	1
CCSK	Sarcoma	1
CCSK	RTK	1

CMN congenital mesoblastic nephroma

Outcome ($n = 210$)

One hundred and seventy-seven (84.3%) of the patients with WT for whom survival data were calculated in this study are alive and 33 (15.7%) have died. Of these, 23 died from the tumor and 7 died as a result of their treatment. Figure 1 shows the OS and RFS curves for patients with WT, CCSK, and RTK. The 5-year OS and RFS rates were 91.1 and 82.0% for WT ($n = 155$), 74.5 and 72.9% for CCSK ($n = 15$), and 22.2 and 16.7% for RTK ($n = 18$), respectively. The prognosis of patients with RTK was significantly worse than that of patients with WT or CCSK.

Figure 2 shows the OS and RFS curves for 132 patients with WT according to the clinical stage defined by the Japanese Staging System. Five-year OS and RFS rates were 90.5 and 86.8% for stage I ($n = 54$), 92.2 and 72.1% for stage II ($n = 43$), 90.9 and 66.4% for stage III ($n = 11$), 86.7 and 58.4% for stage IV ($n = 15$), and 78.7 and 78.7% for stage V ($n = 12$), respectively. RFS rates

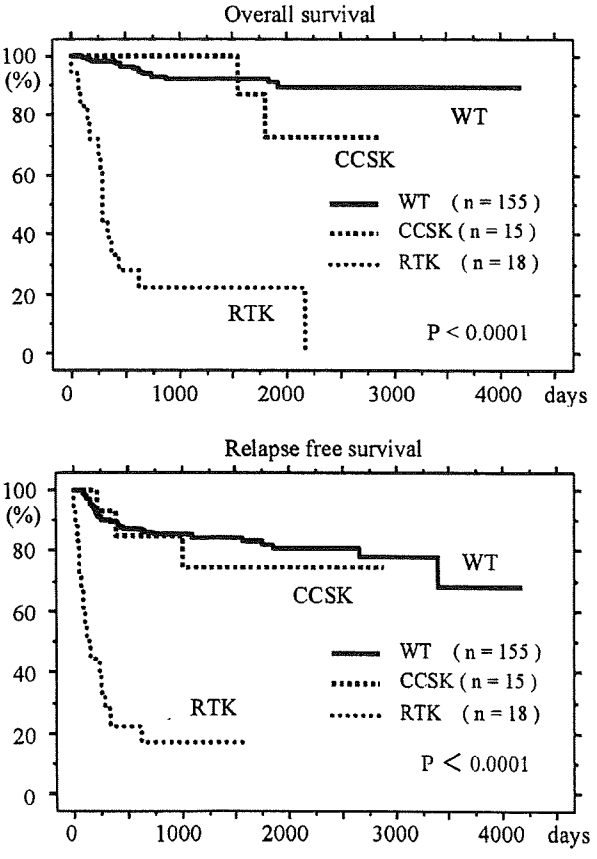

Fig. 1 Overall survival (*upper*) and relapse-free survival (*lower*) curves for patients with Wilms tumor (WT), clear cell sarcoma of the kidney (CCSK), and rhabdoid tumor of the kidney (RTK)

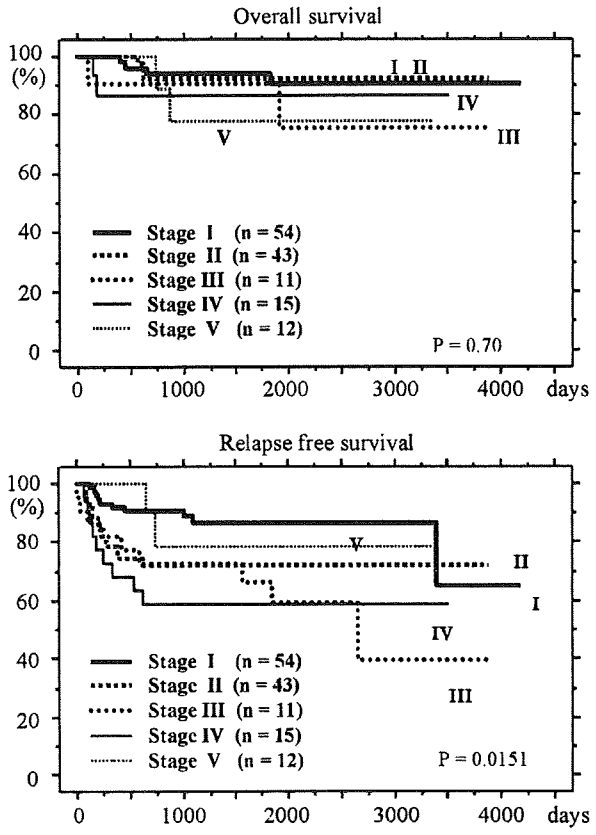

Fig. 2 Overall survival (*upper*) and relapse-free survival (*lower*) curves for patients with Wilms tumor according to the clinical stage classified according to the Japanese Staging System defined by the Japanese Society of Pediatric Surgeons

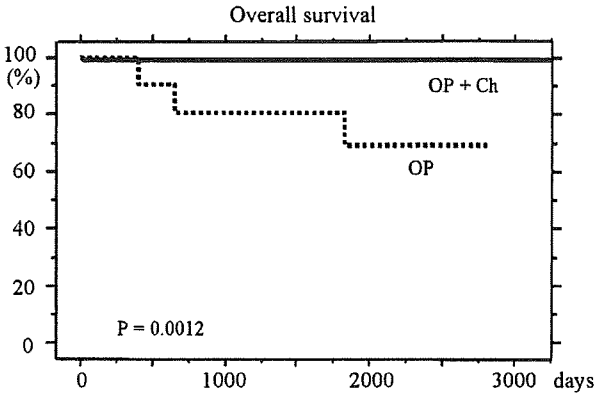

Fig. 3 Overall survival curves for patients with stage I Wilms tumor treated with surgery only and treated with surgery and postoperative chemotherapy using protocol EE-4A. The prognosis of the cases treated with surgery only was significantly worse than those treated with surgery and postoperative chemotherapy

were significantly lower in advanced cases; however, the differences in OS rates were not significant. Table 4 lists the six patients with WT who showed anaplastic histology; they are all alive without disease. Twenty-five out of 155 patients with WT relapsed, and of these 16 re-entered complete remission following salvage treatment.

Between 1996 and 2003 patients under 2 years of age with stage I, favorable histology nephroblastoma, and whose tumor weighed <550 g, received no postoperative chemotherapy. However, 4 cases out of 11 had recurrent disease. The prognosis of cases with stage I WT treated with surgery only was significantly worse than for those treated with surgery and postoperative chemotherapy (protocol EE-4A) (Fig. 3). Therefore from January 2003, the protocol was changed so that postoperative chemotherapy was performed in all cases.

Japan Wilms Tumor Study recommendations for the management of bilateral WT include initial biopsy or tumor resection and local staging followed by chemotherapy according to abdominal stage and histologic features, and second-look surgery at week 5. The surgeon should attempt to preserve renal function without compromising cancer control; 10 of the 12 patients with bilateral WT in this trial are alive. Concerning renal preservation, nephrectomy and partial resection of the opposite site was performed in seven cases and bilateral nephrectomy was performed in one case. Finally, two patients experienced renal failure and required dialysis or renal transplantation.

Table 4 Nephroblastoma with anaplasia

Case	Age, sex	Stage	Type	Treatment	Relapse	Prognosis
1	2 years, F	?	Diffuse	?	–	Alive, 3 years
2	6 years, F	I	Diffuse	EE-4A	–	Alive, 8 years
3	5 months, F	I	Focal	Operation only	–	Alive, 5 years
4	3 years, M	III	Diffuse	DD-4A + RT	–	Alive, 6 years
5	3 years, M	II	Focal	DD-4A + RT	+	Alive, 2 years
6	3 years, F	IV	Focal	?	–	Alive, 2 years

F female, *M* male, *RT* radiation therapy

Discussion

The main objective of the JWiTS-1 study was to elucidate the efficacy of the regimen established by the NWTS group for Japanese patients, and to improve the prognosis and quality of life for Japanese children with renal tumors. Prior to the JWiTS survival rates for stages III and IV WT were 81.8 and 57.1%, respectively, 10–15% worse than in Western countries. The staging system used in JWiTS-1 study was similar to the staging system used in the NWTS studies. Therefore, most of the patients were classified into the same stage in both staging systems. In the JWiTS-1 study, 5-year OS rates for stages III and IV WT have been improved to 90.9 and 86.7%. These results were not worse than those from the NWTS-4 or SIOP 93-01 regimens [9, 10]. Therefore, the JWiTS-1 study has successfully achieved its objectives. However, there are some remaining problems to solve.

Histologic characteristics are the most powerful prognostic indicators for renal tumor in children. Previous studies have proven that the prognosis of anaplastic WT, CCSK, and RTK is worse, resulting in their categorization as having "unfavorable histology" [11]. However, the histological diagnosis of WT with focal or diffuse anaplasia, CCSK, or RTK can be difficult. In the present study, around 5% of the children with "unfavorable histology" had an initial institutional diagnosis of favorable WT (Table 3). These distinctions are critical, because they result in the administration of different chemotherapy regimens. Thus, prompt central pathological review is essential to ensure that all children have an accurate diagnosis and receive appropriate treatment in all cases. Therefore, central diagnosis is considered to be important in improving outcomes for children with renal tumor.

A retrospective study of pathology samples from the NWTS-1 trial showed that anaplasia (irregular mitotic figures, large nuclear size, and hyperchromasia) is associated with worse prognosis [11]. The results from NWTS studies have shown that anaplasia is present in about 5% of WT cases, and that it is rare in patients younger than 2 years and increases to 13% in those over 5 years old. In the present Japanese study, anaplasia was present in 6 of 172 patients with WT (3.5%), and all of them are alive. Our results suggest that the incidence of anaplastic histology is a little lower and that the biology is more favorable in Japanese patients with WT than in Western populations. However, the number of cases in this trial is too small to draw further meaningful conclusions.

Cell sarcoma of the kidney and RTK initially believed to belong to the WT family, are now considered distinct tumor types. In the present study, 5-year OS rates were 91.1% for WT, 72.9% for CCSK, and 22.2% for RTK. Therefore, the prognosis was extremely worse in RTK those in WT and CCSK. There results were compatible with the recent studies conducted by the NWTS [12–15]. In the NWTS-4 study, the 4-year OS rate was only 23.3% in patients with RTK despite aggressive postoperative chemotherapy. Therefore, the development of more effective protocols for RTK is an urgent issue for the future program of the JWiTS.

Retrospective review of NWTS-4 found that a quite favorable outcome occurred in patients younger than 2 years, with tumors of <550 g, and with stage I favorable histology [16]. Therefore, from the outset the JWiTS-1 protocol included no adjuvant treatment after nephrectomy for these patients. However, the protocol was changed in 2003 because of the high-relapse rate. Figure 3 compares the survival of patients with stage I WT treated with surgery only and surgery followed by chemotherapy regimen EE-4A. The prognosis for patients who received postoperative chemotherapy was significantly better than for patients treated by surgery only. The result demonstrated that postoperative chemotherapy could effectively eradicate undetected residual disease in patients with stage I WT, and therefore, we changed the protocol so that every patient now receives postoperative chemotherapy.

Our recommendations for the management of bilateral WT include initial tumor resection or biopsy followed by chemotherapy and second-look surgery at week 5. Long-term survival rates for patients with synchronous bilateral WTs are reported to be 70–80% [17–19]. These results are compatible with ours, in which the 5-year OS rate for patients with bilateral WT was 78.7%. Bilateral WT poses the challenge of establishing local tumor control while preserving renal function.

With regard to renal preservation, among the 15 cases with bilateral WT hemilateral nephrectomy was performed in seven cases and bilateral nephrectomy was performed in one. Finally, two patients had renal failure and needed dialysis or renal transplantation. These results were not satisfactory, and in response to this the protocol for the treatment of bilateral tumor will be changed in the near future, so that preoperative chemotherapy will be performed to shrink tumors without biopsy confirmation. Preoperative chemotherapy often results in a significant reduction in tumor size, thereby facilitating subsequent renal salvage.

To avoid acute and long-term toxicities, therapy should be reduced for children with low-risk tumors. For this reason, reliable biological prognostic markers are needed to distinguish between favorable and unfavorable tumors. There are few prognostic factors for pediatric renal tumor, but several biological factors have been identified recently. One such factor is loss of heterozygosity (LOH) on chromosomes 1p and 16q [20]. The NWTS-5 study has shown that LOH on the 16q and 1p chromosomal arms is associated with an adverse prognosis regardless of tumor

stage and histology. The study also showed positive links between telomerase RNA expression and relapse rates, although there was no association with overall survival [21]. During the JWiTS study, we created a tissue bank to help elucidate molecular biological mechanisms operating in pediatric renal tumor. Using these materials, several lines of basic research have been pursued on the biological markers associated with WT and the roles of the *IGF2* and *WT1* genes in the tumorigenesis of WT have been clarified [22–24].

In conclusion, the JWiTS-1 treatment protocol has provided a reasonable standard of care for patients with WT and CCSK. However, the prognosis for patients with RTK and bilateral and relapsed WT is not satisfactory. We have to seek more effective therapy for patients with these high-risk tumors in the future programs of the JWiTS.

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Two Candidate Tumor Suppressor Genes, *MEOX2* and *SOSTDC1*, Identified in a 7p21 Homozygous Deletion Region in a Wilms Tumor

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A SNP-based array analysis of 100 Wilms tumors (WT) from 97 patients identified 7p alterations (hemizygous and homozygous deletions and uniparental disomy) in nine tumors. The homozygous deletion (HD) region of 7p21 found in one tumor partially overlapped with another HD region reported previously, and was narrowed down to a 2.1-Mb region. Based on an expression analysis of 10 genes located in the HD region in 3 WT lines and previous studies on tumorigenic roles of *MEOX2* and *SOSTDC1*, we further analyzed these two genes. Sequencing showed no mutation in *MEOX2*, but two missense mutations (L50F and Q129L) in *SOSTDC1* in four tumors; L50F in two tumors was of germline origin. Expression levels (0, 1+ and 2+) of *MEOX2* were lower in four tumors with 7p alterations than in 18 tumors with no 7p alterations ($P = 0.017$), and those of *SOSTDC1* tended to be lower in five tumors with 7p alterations or *SOSTDC1* mutation than in 17 tumors with no 7p alterations or *SOSTDC1* mutation ($P = 0.056$). There were no significant differences in clinical characteristics between nine patients with 7p alterations and 88 patients with no 7p alterations; however, there was a difference in the status of *IGF2* (uniparental disomy, loss of imprinting, or retention of imprinting) between the two patient groups ($P = 0.028$). Losses of *MEOX2* and *SOSTDC1* may accelerate angiogenesis and augment signals in the Wnt pathway, respectively. Both genes may be prime candidates for 7p tumor suppressor genes, which may have a role in the progression of Wilms tumorigenesis.

INTRODUCTION

Wilms tumor (WT) (OMIM 194070) is one of the most common pediatric malignancies, accounting for 8% of childhood cancers and occurring in 1 in 10,000 children. The development of WT has been associated with abnormalities of genes or chromosomal regions, which are involved in the development of the embryonic kidney. The abnormalities include *WT1* located at 11p13, *IGF2*, and *H19* at 11p15.5, *WTX* at Xq11, 16q (*WT3*), 17q12-q21 (*WT4*), and 7p21-p11.2 (*WT5*) (Perlman et al., 2004; Rivera and Haber 2005; Rivera et al., 2007); however, deletion or mutation of *WT1* and *WTX* has been found in only 15–33% and 7–24%, respectively (Haruta et al., 2008; Perotti et al., 2008; Ruteshouser et al., 2008; Fukuzawa et al., 2009), and loss of imprinting (LOI) of *IGF2* in 40–70% of tumors (Ravenel et al., 2001; Yuan et al., 2005). Although WT is thought to arise according to the original paradigms of Knudson's two-hit model (Knudson and Strong, 1972), it has become apparent that several known and unknown genetic events also contribute to Wilms tumorigenesis.

Previous cytogenetic, loss of heterozygosity (LOH), and comparative genomic hybridization (CGH) studies indicated alterations of the short arm of Chromosome 7 in a substantial number (10–25%) of WTs (Wang-Wuu et al., 1990; Kaneko et al., 1991; Grundy et al., 1998; Powlesland et al., 2000; Sossey-Alaoui et al., 2003; Yuan et al., 2005), and suggested the presence of tumor suppressor genes in the deleted region. The *POU6F2* gene at 7p14.1 (Perotti

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et al., 2004) and *PTH-B1* at 7p14.3 (Vernon et al., 2003) have been proposed as 7p (*WT5*) genes, however; the roles of the two genes in Wilms tumorigenesis have not been fully elucidated. In addition, previous studies proposed another 7p region (7p22-p15) where candidate 7p genes reside (Grundy et al., 1998; Powlesland et al., 2000).

The single nucleotide polymorphism (SNP) array is a newly developed tool that can detect hemizygous and homozygous chromosomal deletions and uniparental disomy (UPD), and is superior to previous microsatellite markers for detecting LOH that could not distinguish deletions from UPD (Nannya et al., 2005; Yuan et al., 2005; Haruta et al., 2008). The detection of a small homozygous deletion (HD) has been crucial for the identification of tumor suppressor genes (Gessler et al., 1990), and use of the SNP array is the most suitable way to detect such a deletion (Northcott et al., 2009). An analysis of the present series of 100 WTs showed 7p loss or UPD in nine tumors, one of which had a HD region of 4.3 Mb at 7p21.3-p21.1. The region overlaps with a homozygously deleted region reported in a WT by Sossey-Alaoui et al., (2003), and was narrowed down to an area of 2.1 Mb. Of the eight genes within the HD region, we identified two, *MEOX2* (mesenchyme homeobox 2) and *SOSTDC1* (sclerostin domain-containing-1), as candidate tumor suppressor genes involved in Wilms tumorigenesis.

MATERIALS AND METHODS

Patients and Samples

One hundred WTs were obtained from 97 Japanese infants or children; three pairs of tumors were obtained from three patients with bilateral WTs. Eight patients with malformations were syndromic; two with Drash syndrome, five with WAGR syndrome and one with urinary tract malformation, and all three patients with bilateral tumors were syndromic. The remaining 89 patients had no malformations and a unilateral sporadic tumor. Two (Nos. 1 and 100) of the 89 patients were identical twins and their tumors shared the same frameshift mutation in Exon 1 of *WT1*; one (No. 1) was reported as No. 36 in the paper by Haruta et al., (2008). The age at diagnosis ranged from 2 months to 15 years with a median of 2 years and 9 months. Tumors were staged at the time of initial biopsy or surgery, which was carried out between September 1986 and September 2004, according to the National Wilms' Tumor Study Group (NWTS) staging system (d'Angio et al., 1989).

In all cases, the diagnosis of WT was made with routine hematoxylin- and eosin-stained slides by pathologists at each institution or the JWiTS pathology panel according to the classification proposed by the Japanese Society of Pathology and/or the NWTS pathology panel (Beckwith and Palmer, 1978; Japanese Society of Pathology, 2008). Pathologists in each institution verified that the sample for molecular genetic analysis contained 70% or more tumor cells. Three cell lines derived from Wilms tumors (HFWT, Ishiwata et al., 1991; WiT49, Alami et al., 2003; and CCG99-11, a gift from Dr. Benjamin Tycko) were included for mutation, expression, and SNP array analyses of the candidate genes. Normal samples were obtained from either peripheral blood or normal renal tissue adjacent to the tumor from 21 of 97 patients. The status of *WT1*, *IGF2*, and *CTNNB1* was analyzed as previously reported (Haruta et al., 2008). The ethics committee of Saitama Cancer Center approved the study design.

Copy Number and LOH Analysis Using SNP Arrays and Quantitative Real-Time PCR

High-resolution SNP arrays, Affymetrix Mapping 50K-*Xba* and 250K-*Nsp* arrays (Affymetrix, Santa Clara, CA), were used to analyze the chromosomal copy number and LOH status in 100 tumors as described previously (Nannya et al., 2005; Haruta et al., 2008). The analysis detected a HD at 7p21 in one tumor (No. 1), and the copy number of six genes within or close to the HD region was validated in this tumor by real-time quantitative PCR using a LightCycler (Roche Diagnostics, Indianapolis, IN). Primers and probes designed to specifically amplify the six genes or a reference gene, *MOCS2*, at 5q11 are listed in Table 1.

Mutation Analysis of the *MEOX2* and *SOSTDC1* Genes

To detect point mutations and deletions of the two candidate genes, *MEOX2* and *SOSTDC1*, genomic DNA from each tumor sample or cell line was amplified using four or two sets of primers (Table 2). PCR products were directly sequenced with the BigDye Terminator v3.1

TABLE 1. Primer Sequence, Genomic Position, PCR Condition, and Product Size[a]

Primer name	Primer sequence	Genomic position	Annealing temp. (°C)	Product size (bp)
ARL4A 3′-F	TCTTCTCCCTACCCCACAAA	Exon 1	58	99
ARL4A 3′-R	TTCAGACCAAATTCCCACAAC			
TaqMan probe	TGGGGAAG			
ETV1 5′-F	GCGATCCATCAGTTTGGATT	Upstream of exon 1	58	63
ETV1 5′-R	TCAATTCGGTGGTTTTTCCT			
TaqMan probe	TGGAGGAG			
ETV1 3′-F	CACGTTTTTGGCTTTTTCCT	Intron 11	58	63
ETV1 3′-R	AGAAAAACCCATCCTCACCA			
TaqMan probe	CTTCCCCA			
DGKB 5′-F	TTCCCATTCTCTTTCACTTGTGT	Upstream of exon 1	58	60
DGKB 5′-R	TTTCTCTCCCAAGGGCAAC			
TaqMan probe	CTTCCCCA			
DGKB 3′-F	CATTCCCATAGATTTTTACATCTCC	Exon 25	58	74
DGKB 3′-R	CGGGTTTTTCTCACAGGTTAGT			
TaqMan probe	CTTCCCCA			
MEOX2 5′-F	AAAAAGTGACAGAGGGTGGTG	Exon 1	58	78
MEOX2 5′-R	TCCATAGCATGCAAGTTTCG			
TaqMan probe	CTTCCCCA			
MEOX2 3′-F	AAGGAACTGGTGAATGTGAAAAA	Exon 3	58	88
MEOX2 3′-R	AGTCCCCTGTTTGCTGGAG			
TaqMan probe	GGGAGAAG			
SOSTDC1 5′-F	CCGAATGTTAACTAGATTCAGGAAA	Upstream of exon 1	58	70
SOSTDC1 5′-R	GCAGGCGTATTCTATATCAACGA			
TaqMan probe	CAGAGGAG			
SOSTDC1 3′-F	TGCCAGTGCTCCCTAACTG	Exon 2	58	64
SOSTDC1 3′-R	GAGCTCCTCCTGCTCCAGTA			
TaqMan probe	TGGAGGAG			
TSPNA13 5′-F	CAACCTGCTTTACACCGTGA	Exon 1	58	76
TSPNA13 5′-R	AAGAGCTCCCAAGCAGGTG			
TaqMan probe	TCCTGCTC			
TSPNA13 3′-F	CACGGTGTCTTCTCTCCATGT	Exon 6	58	65
TSPNA13 3′-R	ACAGGGGGACACATATGACG			
TaqMan probe	CTTCCTGC			
MOCS2 (5q11) –F	CAGCAAACCACATACACTTTATCA	Intron 3	58	71
MOCS2 (5q11) –R	TGAGATTTGTTTTTCTTAATCAGTC			
TaqMan probe	TGGGGAAG			

[a]Primers for real-time PCR analysis of 6 genes within or close to the homozygous deletion region at 7p21.

TABLE 2. Primer Sequence, Genomic Position, PCR Condition, and Product Size[a]

Primer name	Primer sequence	Genomic position	Annealing temp. (°C)	Product size (bp)
MEOX2 ex 1-1F	AGAAGTGCACCGCTATTGCT	Exon 1	60	533
MEOX2 ex 1-1R	CCAAGCTGGAAGAGTTGGAG			
MEOX2 ex 1-2F	CTTGCATAATCGCGGGATAC	Exon 1	60	495
MEOX2 ex 1-2R	GGCAACACATTCCCATCTTC			
MEOX2 ex 2F	TGTCTAGCGCAGTACCTGGA	Exon 2	60	425
MEOX2 ex 2R	TGGCAAAATTTTATCATGGA			
MEOX2 ex 3F	GCCTTTGAGGAACTTCTTTTCA	Exon 3	60	459
MEOX2 ex 3R	CAGATTCGAAATGCCTGGAT			
SOSTDC1 ex 1F	AGGGATCCCACCCCTTCT	Exon 1	60	550
SOSTDC1 ex 1R	TACCTAGGGAAGAATGCCAAC			
SOSTDC1 ex 2F	ATTTTTGTCATTGCAGATCACTTT	Exon 2	60	580
SOSTDC1 ex 2R	CAGTGGCAGGCTTGAGTCTT			

[a]Primers for mutation analysis of *MEOX2* and *SOSTDC1*.

Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Reverse-Transcription (RT)-PCR Analysis of 10 Genes Within the HD Region at 7p21

We performed a RT-PCR analysis to investigate the expression status of 10 genes within the 7p21 HD region in tumor samples with or without 7p alterations (loss and UPD), three cell lines, and fetal and normal kidney tissues. Primers, their locations, and PCR conditions are listed in Table 3. Because the three cell lines and fetal and normal kidney tissues did not express *LOC100131022* and *LOC100129335*, we used genomic DNA of normal human leukocytes as a control. The results of the RT-PCR analysis for *MEOX2* and *SOSTDC1* were treated semi-quantitatively, and defined as undetectable (0), lower expression levels than in fetal kidney tissues (1+), and comparable expression levels to fetal kidney tissues (2+).

Bisulfite Treatment and Methylation-Specific PCR (MSP) of *MEOX2*

Genomic DNA from tumor samples and cell lines was treated with sodium bisulfite as described previously (Herman et al., 1996; Sugawara et al., 2007). Bisulfite-modified DNA was amplified with primers specific for methylated and unmethylated sequences from three CpG islands within or upstream of *MEOX2* Exon 1 (CGI +105, CGI −270, and CGI −609) (Fig. 1). The primer sequences and their locations in the original genomic sequences are listed in Table 4. There were no CpG islands within or upstream of *SOSTDC1* Exon 1, and MSP was not possible for the gene. CpGgenome™ Universal methylated DNA (Chemicon International,

TABLE 3. Primer Sequence, Genomic Position, PCR Condition, and Product Size[a]

Primer name	Primer sequence	Genomic position	Annealing temp. (°C)	Product size (bp)
LOC100131022-F	GAGGGCTATTATGGGCTGAA	Exon 1	58	223
LOC100131022-R	TCCCTGTGTCTCTCTGTCCA			
ETV1-F	TACCCCATGGACCACAGATT	Exons 6–7	60	168
ETV1-R	CACTGGGTCGTGGTACTCCT			
DGKB-F	CATGGTAATGGTGTGCTTGC	Exons 2–5	60	219
DGKB-R	CAGACCGCCTGATAGGAGAG			
TMEM195-F	GGGTTCATCATGGCAGAAAT	Exons 6–9	60	203
TMEM195-R	AGGTGTGGCCCAGAATGTAG			
MEOX2-F	GTCAGAAGTCAACAGCAAACCCAG	Exons 2–3	64	286
MEOX2-R	AATTCCCGACAGCTCTGATG			
LOC729920-F	CAGGCCCTGGAGAGAGTATG	Exons 1–2	60	238
LOC729920-R	ATGGTCTCACAGCATCATGG			
SOSTDC1-F	TTCCTCCTGCCATTCATTTC	Exons 1–2	60	202
SOSTDC1-R	GTGTTCCGATCCAGTCCAGT			
LOC100129771-F	ATGGGGATGCGTCTGAATAC	Exons 1–2	60	224
LOC100129771-R	TAGTTTTGCTGTGGCCCTTC			
LOC100129335-F	CCAAGCCAAGTCTCTCTTCG	Exon 2	58	157
LOC100129335-R	CTGTAGGGCAGTTTCGGATG			
ANKMY-F 2-F	ATTGGTTCTGATCCCACTGC	Exons 7–9	60	233
ANKMY-F 2-R	GCCTCCAACTGTTGCTTTTC			

[a]Primers for RT-PCR for 10 genes within or near the homozygous deletion region at 7p21.

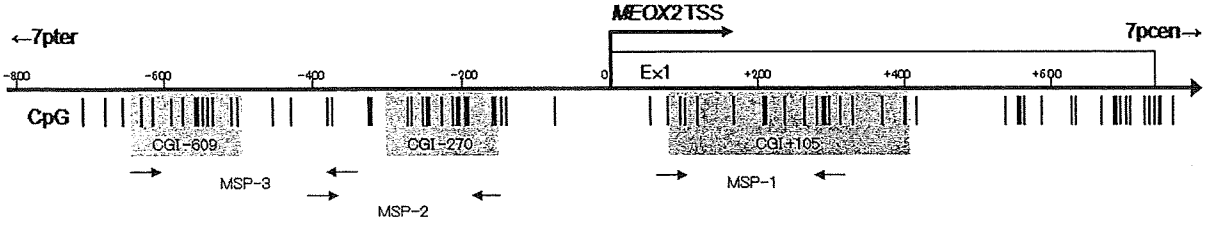

Figure 1. The location of *MEOX2* fragments analyzed by the methylation-specific PCR method. Horizontal arrows indicate the locations of primers, and a bent arrow indicates the transcription start site.