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Cytogenetic and Genome Research

Imprinting disruption of the *CDKN1C/KCNQ1OT1* domain: the molecular mechanisms causing Beckwith-Wiedemann syndrome and cancer

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Abstract. Human chromosomal region 11p15.5, which is homologous to mouse chromosome region 7F5, is a well-known imprinted region. The *CDKN1C/KCNQ1OT1* imprinted domain, which is one of two imprinted domains at 11p15.5, includes nine imprinted genes regulated by an imprinting center (IC). The *CDKN1C/KCNQ1OT1* IC is a differentially methylated region of *KCNQ1OT1 (KCNQ1OT-DMR)* with DNA methylation on the maternal allele and no methylation on the paternal allele. *CDKN1C* (alias $p57^{KIP2}$), an imprinted gene with maternal expression, encoding a cyclin-dependent kinase inhibitor, is a critical gene within the *CDKN1C/KCNQ1OT1* domain. In Beckwith-Wiedemann syndrome (BWS), approximately 50% of patients show loss of DNA methylation accompanied by loss of histone H3 Lys9 dimethylation on maternal *KCNQ1OT-DMR*, namely an imprinting disruption, leading to diminished expression of *CDKN1C*. In cancer, at least three molecular mechanisms – imprinting disruption, aberrant DNA methylations at the *CDKN1C* promoter, and loss of heterozygosity (LOH) of the maternal allele – are seen and all three result in diminished expression of *CDKN1C*. Imprinting disruption of the *CDKN1C/KCNQ1OT1* domain is involved in the development of both BWS and cancer and it changes the maternal epigenotype to the paternal type, leading to diminished *CDKN1C* expression. In this review, we describe recent advances in epigenetic control of the *CDKN1C/KCNQ1OT1* imprinted domain in both humans and mice.

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Imprinting is an epigenetic phenomenon that leads to monoallelic expression of genes in a parent-of-origin-dependent manner. It is thought that most imprinted genes form clusters, or imprinted domains, and are under domain level regulation (Reik and Walter, 2001). Human 11p15.5, which is homologous to mouse 7F5, is a well-known and well-studied imprinted region. It has been shown that this imprinted region is separated into two domains, *CDKN1C/KCNQ1OT1* and *IGF2/H19*, and that each domain is regulated by a functionally independent imprinting center (IC) in both humans and mice (Caspary et al., 1998; Horike et al., 2000; Fitzpatrick et al., 2002). Generally, ICs are differentially methylated regions (DMRs), which show DNA methylation on one parental allele but not on the opposite allele. The ICs for *CDKN1C/KCNQ1OT1* and *IGF2/H19* domains are *KCNQ1OT-DMR* and *H19-DMR*, respectively. Recently, another epigenetic process, namely histone modification, has attracted researchers' attention. Jenuwein and Allis have proposed the 'histone code' hypothesis which states that different modifications of specific histone amino acids or combinations of these, are translated into functionally distinct effects on nuclear processes (Strahl and Allis, 2000; Jenuwein and Allis, 2001). For example, histone H3 Lys9 methylation (H3mK9) is associated with the formation of stably silenced chromatin regions in mammals, whereas acetylation of histone H3 and H4 (H3Ac and H4Ac) and methylation of histone H3 Lys4 (H3mK4) are correlated with transcriptionally active chro-

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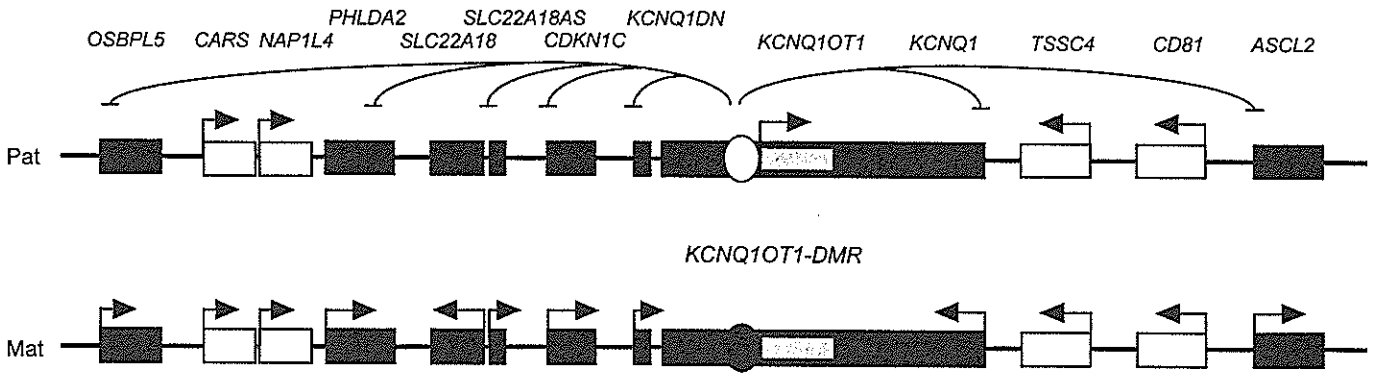

Fig. 1. Human *CDKN1C/KCNQ1OT1* imprinted domain on 11p15.5. Maternally and paternally expressed genes are indicated by red and blue boxes, respectively. DNA methylation status at *KCNQ1OT-DMR* is shown by the white oval (unmethylated) and by the black oval (methylated). Unmethylated *KCNQ1OT-DMR* on the paternal allele (Pat), which works as a silencer and as a promoter for *KCNQ1OT1* RNA transcription, represses the surrounding maternally expressed genes. Methylated *KCNQ1OT-DMR* on the maternal allele (Mat) cannot work as a silencer, and *KCNQ1OT1* RNA cannot be transcribed. As a result, surrounding maternal expressed genes are transcribed.

matin structures. It has been shown that histone modification, as well as DNA methylation, are important for the regulation of imprinted genes (Saitoh and Wada, 2000; Xin et al., 2001; Fournier et al., 2002; Higashimoto et al., 2003; Yang et al., 2003a, b). Since genetic and epigenetic abnormalities of the 11p15.5 imprinted region are found in Beckwith-Wiedemann syndrome (BWS), a tumor predisposing overgrowth syndrome, and in various cancers, the region has been thought to be involved in the development of these diseases.

In this review, we focus on abnormalities in epigenetic mechanisms, such as DNA methylation and histone modification, at the *CDKN1C/KCNQ1OT1* domain in BWS and cancer. We also describe the importance of *CDKN1C*, which is regulated by *KCNQ1OT-DMR*, in these diseases and its imprinting regulation in humans and mice.

CDKN1C/KCNQ1OT1 domain in mouse and human

The imprinted region at 11p15.5 consists of two independent domains, *CDKN1C/KCNQ1OT1* and *IGF2/H19*. The *CDKN1C/KCNQ1OT1* domain extends from *OSBPL5* (alias *OBPH1*) to *ASCL2* (Fig. 1). The structure and the imprinting status of these domains are mostly conserved between humans and mice. In the human *CDKN1C/KCNQ1OT1* domain, eight genes *(OSBPL5, PHLDA2, SLC22A18, SLC22A18AS, CDKN1C, KCNQ1DN, KCNQ1, ASCL2)* are expressed exclusively or preferentially from the maternal allele, whereas *KCNQ1OT1* is exclusively expressed from the paternal allele (Fig. 1) (Reik and Walter, 2001). *KCNQ1OT1* is a non-coding antisense-transcript to *KCNQ1*, and its promoter exists within *KCNQ1OT-DMR* (Lee et al., 1999; Mitsuya et al., 1999; Smilinich et al., 1999). *KCNQ1OT-DMR*, the IC for the *CDKN1C/KCNQ1OT1* domain, shows a pattern of maternal DNA methylation and paternal unmethylation. In mice, *KCNQ1OT-DMR* is methylated in oocytes, but not in sperm (Engemann et al., 2000; Yatsuki et al., 2002). In mice with a paternally inherited deletion of *Kcnq1ot-DMR*, derepression of imprinted genes, which are normally silent on the paternal allele, was evident (Fitzpatrick et al., 2002). Mouse hybrid cells with human paternal chromosome 11 also showed activation of imprinted genes, which are normally silent on the paternal allele, because of a lack of *Kcnq1ot1* expression on the targeted chromosome (Horike et al., 2000). These findings indicate that unmethylated *KCNQ1OT-DMR* represses within the *CDKN1C/KCNQ1OT1* domain on the paternal allele (Fig. 1).

How does *KCNQ1OT-DMR* regulate expression of imprinted genes? In both humans and mice, it has been shown by experiments based on plasmid constructs (Kanduri et al., 2002; Du et al., 2003; Mancini-DiNardo et al., 2003; Thakur et al., 2003) that *KCNQ1OT-DMR* functions as an insulator and/or bi-directional silencer. Recently, Thakur et al. (2004) reported that *KCNQ1OT1* RNA itself possesses a bi-directional silencing property in such plasmid-based systems. Although this mechanism is similar to that of *Air* RNA within the *Igf2r* locus, it is unknown whether the mechanism functions in vivo. Niemitz et al. (2004) reported microdeletion of the *KCNQ1OT1* region in familial BWS. In this family, since there was no phenotype when the deletion was paternally inherited, it was suggested that the *KCNQ1OT1* RNA itself is not necessary for normal development in humans. This finding is inconsistent with the silencing property of *KCNQ1OT1* RNA in mice. When the deletion was maternally inherited, it caused BWS with silencing of *CDKN1C*, suggesting the existence of an enhancer element for *CDKN1C* in the deleted region. This is supported by the experiment in which a site-specific translocation between *Cdkn1c* and *Kcnq1* in mice resulted in loss of expression and loss of imprinting of the genes including *Cdkn1c* (Cleary et al., 2001). Mechanisms governing imprinting of the *CDKN1C/KCNQ1OT1* domain have not been completely elucidated. Thus, further in vitro and in vivo experiments might be necessary to clarify the specific mechanisms involved here.

As for histone modification, histone H3 Lys9 di-methylation (H3m2K9) is abundant on the DNA methylated ma-

ternal *KCNQ1OT-DMR* in both humans and mice, indicating condensed and inactive heterochromatin. On the other hand, H3Ac, H4Ac, and H3 Lys4 di-methylation (H3m2K4) were observed to be abundant on unmethylated paternal *KCNQ1OT-DMR* in both species, indicating open chromatin and active transcription (Higashimoto et al., 2003). The histone modification state at *KCNQ1OT-DMR* was associated with DNA methylation status and the expression of *KCNQ1OT1*. This is consistent with similar observations in other imprinting regions, such as *PWS-IC*, *H19-DMR*, DMR2 of *Igf2r* and DMR of *U2af1l1* (Saitoh and Wada, 2000; Xin et al., 2001; Fournier et al., 2002; Yang et al., 2003a, b).

What is the primary imprint mark involved here? Since primary imprints should be marked during gametogenesis, H3m2K9 is thought to be a candidate because chromosomes in oocytes retain histones, whereas histones are replaced by protamines in sperm. In mouse ES cells lacking the H3K9 methyltransferase, G9a, there are both loss of maternal DNA methylation at *PWS-IC* and biallelic expression of *Snrpn*. In contrast, DNA methyltransferase-deficient ES cells lack DNA methylation at *PWS-IC* but show normal levels of H3m2K9 of *PWS-IC* and preserve monoallelic expression of *Snrpn* (Xin et al., 2003). These findings suggest that the primary imprint mark on maternal *PWS-IC* may be H3m2K9. On the other hand, in a recent study using conditional knock out mice (Kaneda et al., 2004), Dnmt3a, which is a de novo DNA methyltransferase, was reported to be essential for the establishment of maternal and paternal imprints. Although Kaneda et al. (2004) referred to the possibility that other factors might be involved in the establishment of imprinting, their results indicated that DNA methylation is a primary imprint mark for several ICs.

CDKN1C, a key gene in the *CDKN1C/KCNQ1OT1* domain

CDKN1C (alias $p57^{KIP2}$), located within the *CDKN1C/KCNQ1OT1* domain, is an imprinted gene with maternal expression, which has attracted attention as a key gene for BWS and cancer. Since *CDKN1C* encodes a cyclin-dependent kinase inhibitor (CDKI) that belongs to the CIP/KIP family, it is considered to be a putative tumor suppressor gene (Lee et al., 1995; Watanabe et al., 1998). Actually, decreased expression of *CDKN1C* has been observed in sporadic cancers and embryonal tumors, including Wilms' tumor. However, mutations of *CDKN1C* are very rare in embryonal tumors and are not found in sporadic cancers (Chung et al., 1996; Hatada et al., 1996a; Schwienbacher et al., 2000; Shin et al., 2000; Kikuchi et al., 2002; Li et al., 2002; Shen et al., 2003; Soejima et al., 2004; Hoffmann et al., 2005). In rare BWS cases, germ-line point mutations of *CDKN1C* have been identified (Hatada et al., 1996b), and mice with maternally inherited deletions of *CDKN1C* showed some features of BWS (Zhang et al., 1997). These findings indicate that within the *CDKN1C/KCNQ1OT1* domain, *CDKN1C* is a critical gene for BWS and cancers.

Mouse *Cdkn1c* is exclusively expressed from the maternal allele, but human *CDKN1C* is predominantly expressed from the maternal allele and partially from the paternal allele. The DNA methylation status in the *CDKN1C* promoter region is different between humans and mice. In mice, the promoter is unmethylated on the maternal allele, leading to active transcription, whereas it is methylated on the paternal allele, leading to inactive transcription (Yatsuki et al., 2002). On the other hand, in humans, the promoter is unmethylated on both alleles (Chung et al., 1996). As for histone modification, H3m2K9 is abundant within the paternal promoter in mice (Higashimoto et al., unpublished data) (Fig. 2A). However, H3m2K9 was not detected in human lymphoblastoid cells (Higashimoto et al., unpublished data) (Fig. 2B). These DNA methylation and H3m2K9 status distinctions may account for the differences in *CDKN1C* imprinted expression observed between humans and mice. These observations also suggest that some physical differences between the two species exist in terms of regulating *CDKN1C* expression.

Imprinting disruption of the *CDKN1C/KCNQ1OT1* domain and BWS

BWS is a congenital syndrome characterized by pre- and postnatal overgrowth, macroglossia, and anterior abdominal wall defects. Additional but variable complications include organomegaly, hypoglycemia in infancy, hemihypertrophy, genitourinary abnormalities, and, in ~5% of children, embryonal tumors (e.g. Wilms' tumor, hepatoblastoma, rhabdomyosarcoma). BWS has a complex etiology and can arise from paternal uniparental disomy (patUPD) of chromosome 11, paternal duplication of 11p15.5, maternally inherited coding mutations in *CDKN1C*, maternal chromosome rearrangements, and loss of imprinting of *IGF2* with DNA hypermethylation or normal methylation of *H19-DMR* (Weksberg et al., 2003). However, the most common mechanism that results in BWS is loss of DNA methylation of *KCNQ1OT-DMR* on the maternal allele. Loss of DNA methylation was observed in ~50% of BWS patients and strongly correlated with maternal loss of H3m2K9 (Higashimoto et al., 2003). Fibroblast cells from BWS patients with loss of maternal DNA methylation at *KCNQ1OT-DMR* have shown diminished expression of the *CDKN1C* (Diaz-Meyer et al., 2003). H3m2K9 at the *CDKN1C* promoter, however, could not be detected in any samples, irrespective of DNA methylation status at *KCNQ1OT-DMR* (Higashimoto, unpublished data) (Fig. 2B). These findings suggest that H3m2K9 at the *CDKN1C* promoter region is not associated with allelic expression of *CDKN1C* in human, and that histone modification at *KCNQ1OT-DMR*, as well as DNA methylation, is important as an epigenetic factor. Taken together, imprinting disruptions of the *CDKN1C/KCNQ1OT1* domain would cause diminished *CDKN1C*, leading to the development of BWS.

The *CDKN1C/KCNQ1OT1* domain and cancer

Loss of heterozygosity (LOH) of 11p15 occurs frequently in several sporadic cancers, including embryonal tumors and some common adult cancers (Henry et al., 1989; Kiechle-

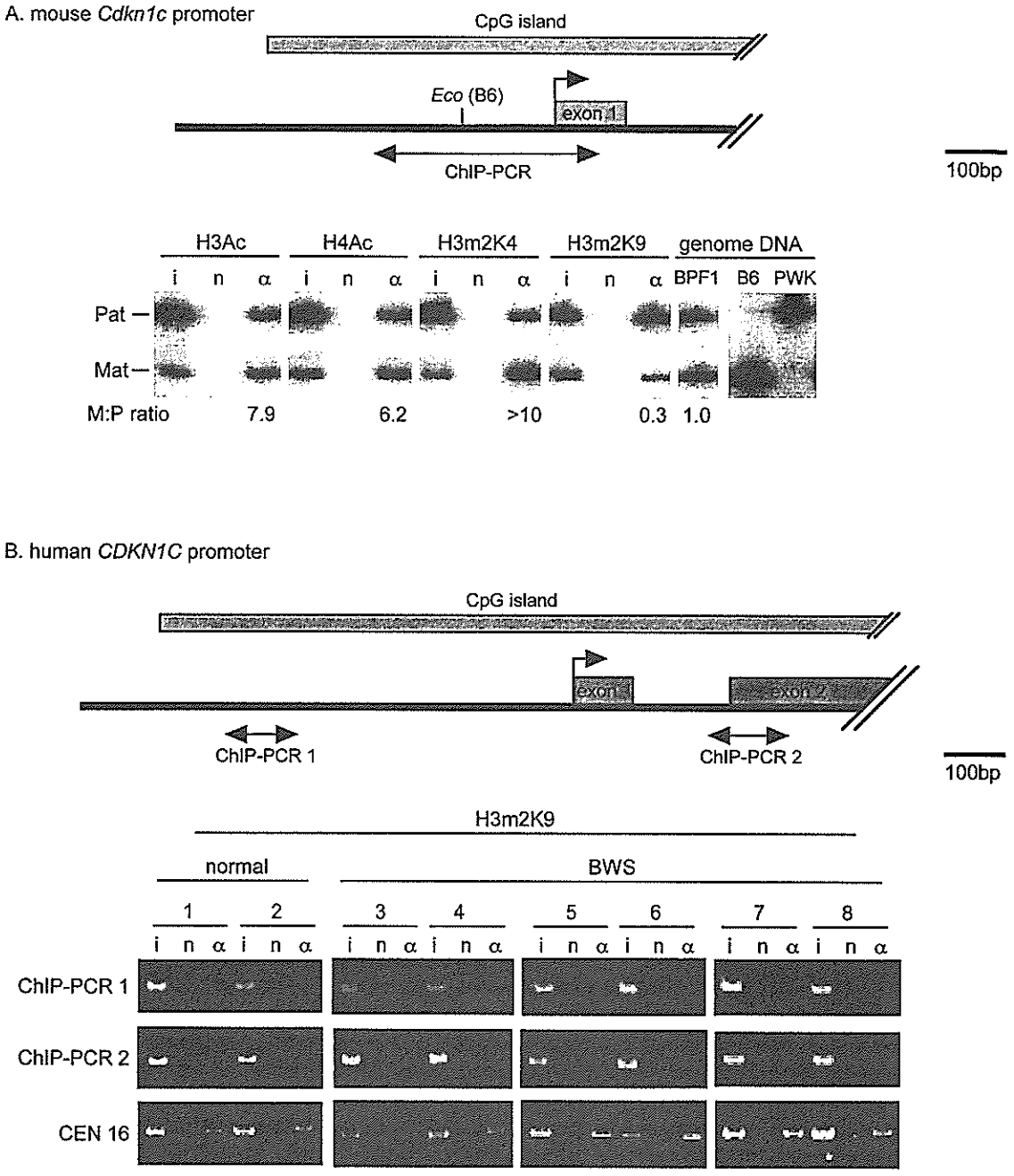

Fig. 2. Histone modification of *CDKN1C* promoter in mouse and human. (**A**) Histone modification at mouse *CDKN1C* promoter. Chromatin of primary fibroblasts derived from (C57BL/6 × PWK)F1 (BPF1) mice was analyzed by chromatin immunoprecipitation (ChIP) with antibodies against H3Ac, H4Ac, H3m2K4, and H3m2K9. ChIP DNAs from fractions representing input (i), no antibody (n), and antibody (α) were subjected to hot-stop PCR. The PCR products were digested with *Eco*O109I and intensity of the PCR products was measured. Ratios of maternal intensity to paternal intensity (M:P), corrected by M:P in the input fraction chromatin, are indicated below each lane. H3Ac, H4Ac, and H3m2K4 are abundant on the maternal allele, whereas H3m2K9 is abundant on the paternal allele. The results of ChIP from reciprocal cross BPF1 fibroblasts were the same (data not shown). These results indicate that histone modification is associated with allelic expression of *Cdkn1c* in mice. *Eco*: a polymorphic *Eco*O109I site in C57BL/6. (**B**) H3m2K9 at the human *CDKN1C* promoter. Lymphoblastoid cells were derived from normal individuals (1 and 2), BWS patients with normal *KCNQ1OT-DMR* (3 and 4), and BWS patients with loss of DNA methylation at *KCNQ1OT-DMR* (5, 6, 7, and 8). ChIP-PCR for ChIP 1 and 2 regions failed to detect H3m2K9 in any sample. The chromosome 16 centromere region was amplified as a positive control for H3m2K9. The results show that H3m2K9 is not associated with allelic expression of *CDKN1C*. The open boxes and broken arrows show exons and the transcription start sites, respectively. A CpG island is indicated by the gray box. Regions analyzed by ChIP-PCR are indicated by double-ended arrows.

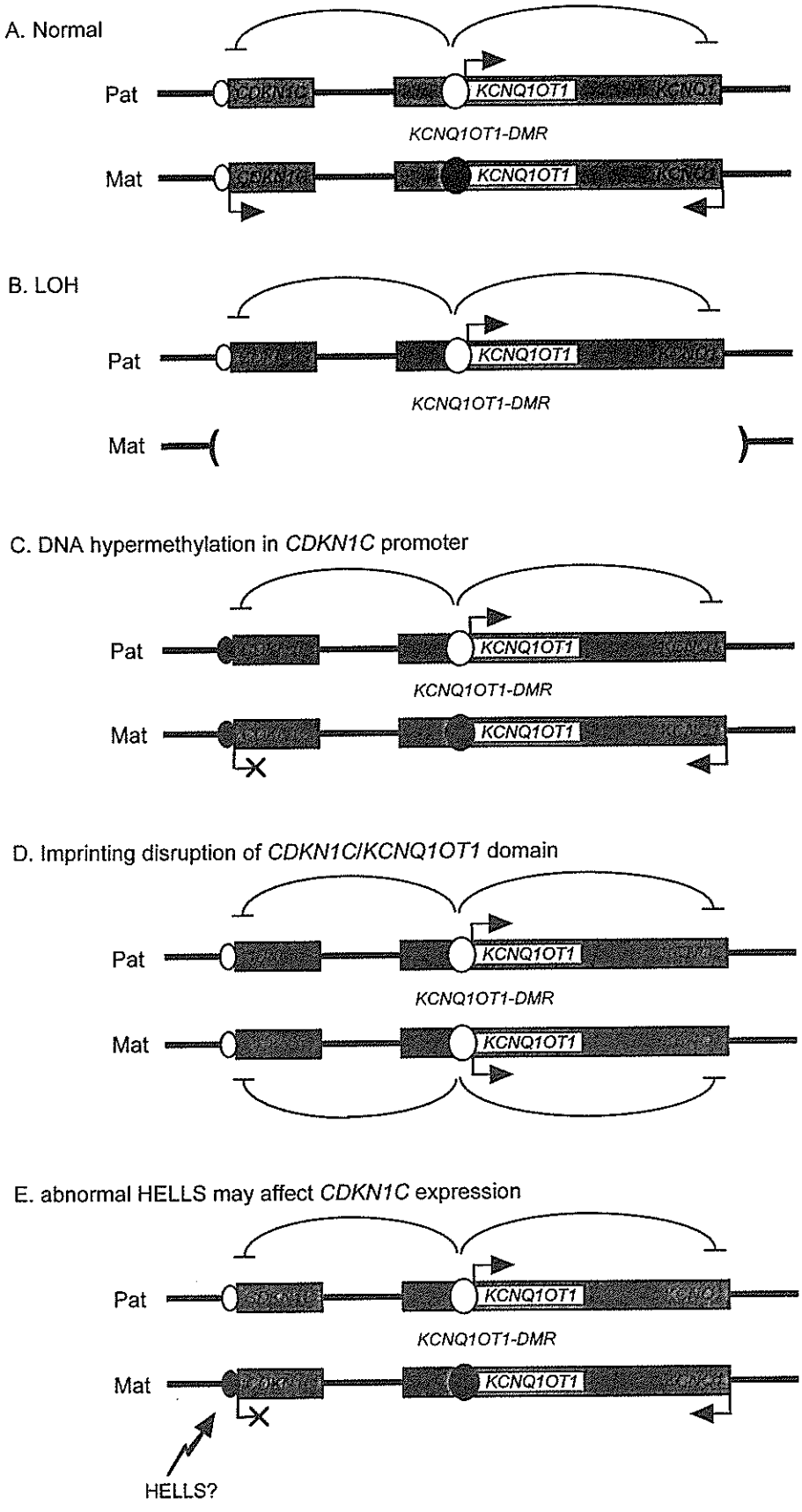

Fig. 3. Possible mechanisms causing diminished expression of *CDKN1C* in cancer. (**A**) Normal situation in the *CDKN1C/KCNQ1OT1* domain. An explanation of the imprinting mechanism is shown in Fig. 1. The *CDKN1C* promoter is unmethylated on both alleles in the normal imprinting state (small white ovals). (**B**) LOH of 11p15.5. The LOH occurs on the maternal allele, where *CDKN1C* is predominantly expressed. (**C**) DNA hypermethylation at the *CDKN1C* promoter. As a result, the *CDKN1C* promoter cannot function. (**D**) Imprinting disruption of the *CDKN1C/KCNQ1OT1* domain. Loss of DNA methylation at *KCNQ1OT-DMR* leads to the repression of the surrounding maternally expressed genes, including *CDKN1C*, on the maternal allele. (**E**) *HELLS* abnormalities may affect *CDKN1C* expression. Because loss of Hells leads to reactivation on the silenced paternal *Cdkn1c* with loss of promoter methylation in mice, it is speculated that over-expression or gain of function mutations of *HELLS* may modulate methylation of the *CDKN1C* promoter. DNA methylated regions are shown by black ovals. Pat: paternal allele; Mat: maternal allele.

Schwartz et al., 1993; Bepler and Garcia-Blanco, 1994; Baffa et al., 1996). The lost allele is commonly maternal. Approximately 5% of BWS patients have embryonal tumors. These findings suggest that at least one maternally expressed gene in 11p15.5 functions as a tumor suppressor. To date, only *CDKN1C* has been identified as a candidate tumor suppressor in the *CDKN1C/KCNQ1OT1* domain. Although no somatic mutations of *CDKN1C* have been found in sporadic cancers, *CDKN1C* expression is decreased in various tumors (Chung et al., 1996; Hatada et al., 1996a; Schwienbacher et al., 2000; Shin et al., 2000; Kikuchi et al., 2002; Li et al., 2002; Shen et al., 2003; Soejima et al., 2004; Hoffmann et al., 2005).

At least three mechanisms may be responsible for the decreased expression of *CDKN1C* (Fig. 3). The first is maternal LOH of 11p. Maternal LOH causes diminished expression of *CDKN1C* because it is expressed predominantly from the maternal allele (Fig. 3A, B). It has been reported that the expression of *CDKN1C* was diminished in Wilms' tumors with LOH less than that in normal fetal kidney and that *CDKN1C* was down-regulated by LOH in some bladder cancer cell lines (Chung et al., 1996; Hoffmann et al., 2005). The second mechanism is DNA hypermethylation at the *CDKN1C* promoter region (Fig. 3C). It is well known that DNA hypermethylation of promoters contributes to silencing of tumor suppressor genes in human cancers (Jones and Laird , 1999). Hypermethylation at the *CDKN1C* promoter region was reported in various adult tumors, but not in Wilms' tumor (Chung et al., 1996; Shin et al., 2000; Kikuchi et al., 2002; Li et al., 2002; Shen et al., 2003; Hoffmann et al., 2005; Satoh and Soejima, unpublished data). These findings suggest that there are differences in the frequency of *CDKN1C* promoter hypermethylation among tumor types. The last mechanism is 'Imprinting disruption', that is loss of DNA methylation at *KCNQ1OT-DMR* on the maternal allele (Fig. 3D). Scelfo et al. (2002) reported that aberrant methylation occurs at *KCNQ1OT-DMR* in various adult tumors. Weksberg et al. (2001) reported that not only patUPD of 11p15 and *H19-DMR* hypermethylation but also loss of methylation at *KCNQ1OT-DMR* are all associated with embryonal tumor development in BWS. We have previously shown that diminished *CDKN1C* expression was correlated with loss of DNA methylation at *KCNQ1OT-DMR* in esophageal cancer, but that it was not correlated with DNA methylation within its own promoter (Soejima et al., 2004). A similar result was reported in some bladder cancers (Hoffmann et al., 2005). Furthermore, loss of H3m2K9 was accompanied by loss of DNA methylation at *KCNQ1OT-DMR*. At the *CDKN1C* promoter region, however, H3m2K9 was scarcely detectable in any esophageal cancers, regardless of DNA methylation status at *KCNQ1OT-DMR* (Soejima et al., 2004). These findings are the same as the results observed for the *CDKN1C/KCNQ1OT1* domain in BWS as described in the preceding section.

However, all cancers showing diminished *CDKN1C* expression cannot be explained by the three mechanisms mentioned above, suggesting the existence of other mechanism(s). For example, Hells (alias Lsh), a member of the SNF2 family of chromatin remodeling proteins, controls DNA methylation in mice. Loss of Hells leads to reactivation on the silenced paternal allele of *CDKN1C*, and it correlates closely with loss of DNA methylation at the promoter on the paternal allele. However, *KCNQ1OT-DMR* and other DMRs were not significantly changed in the mouse embryo (Fan et al., 2005). This suggests the existence of a trans-factor for *CDKN1C* regulation separate from the effects exerted by *KCNQ1OT-DMR*. Abnormalities involving human HELLS, e.g. over expression or gain of function mutations, may influence the imprinting status of *CDKN1C*, although human *CDKN1C* expression is not dependent on promoter methylation. Further studies will be necessary to elucidate the mechanisms for the diminished expression of *CDKN1C*.

Conclusions and perspective

Loss of DNA methylation and of H3K9 methylation at *KCNQ1OT-DMR* causes imprinting disruptions of the *CDKN1C/KCNQ1OT1* domain, changing the maternal epigenotype to the paternal one. It can lead to reduced *CDKN1C* expression and is involved in approximately 50% of BWS patients and in certain cases of cancer. However, many questions remain to be solved. For example, why and how are specific sequences differentially methylated as ICs during gametogenesis to establish imprinting? How is imprinting maintained in somatic cells? What are the molecular mechanisms that transmit the imprint signal from the IC to target genes? Further experiments are required to answer these questions.

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Association of 11q Loss, Trisomy 12, and Possible 16q Loss with Loss of Imprinting of Insulin-Like Growth Factor–II in Wilms Tumor

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We evaluated the *WT1* and *IGF2* status and performed chromosome and/or comparative genomic hybridization analysis in 43 tumor samples from patients with Wilms tumor. On this basis, we classified them into 4 groups: *WT1* abnormality, loss of heterozygosity (LOH) of *IGF2*, loss of imprinting (LOI) of *IGF2*, and retention of imprinting (ROI) of *IGF2*, which were seen in 12%, 30%, 16%, and 42% of the tumors, respectively. Patients in the LOI group were older than those in other groups ($P < 0.01$), and tumors in the *WT1* group had fewer cytogenetic changes than did those in the other groups ($P < 0.01$). It was found that 11q− and +12 were more frequent in the LOI group than in the *WT1*+LOH+ROI group ($P < 0.01$ and $P < 0.01$). There was no difference in the incidence of 16q− between the LOI group and the other groups; however, when we excluded 16 tumors with LOH on 11p15, 16q− tended to be more frequent in the LOI group than in the *WT1*+ROI group ($P = 0.06$). The association of 11q− or +12 with LOI of *IGF2* found in the present study suggests that many tumors with no *WT1* abnormalities need overexpression of *IGF2* together with biallelic inactivation of the tumor-suppressor gene on 11q and/or overexpression of growth-promoting genes on chromosome 12. The 11q gene may code for one of the proteins that constitute a CTCF insulator complex, and its mutation, deletion, or haploinsufficiency may cause insulator abnormalities that might lead to LOI of *IGF2*. © 2006 Wiley-Liss, Inc.

INTRODUCTION

Wilms tumor is the most common kidney tumor in childhood. A tumor-suppressor gene, *WT1*, was isolated in the 11p13 chromosomal region, but deletion or mutation has been found in only 15%–20% of Wilms tumors (Huff, 1998; Nakadate et al., 2001). Loss of imprinting (LOI) of insulin-like growth factor–II (*IGF2*), a paternally expressed gene at 11p15.5, has been reported to occur in 40%–70% of tumors (Ogawa et al., 1993; Rainier et al., 1993), and it was associated with a pathological subtype that occurs in a later stage of renal development (Ravenel et al., 2001). Several studies found the type of loss of heterozygosity (LOH) on 11p that is always caused by loss of the maternal chromosome in 30%–40% of tumors investigated (Schroeder et al., 1987; Grundy et al., 1994; Nakadate et al., 2001). LOI or LOH of *IGF2* may cause overexpression of a gene that gives tumor cells a growth advantage or modifies their differentiation stage (Sakatani et al., 2005), and *IGF2* is the primary candidate for being the *WT2* gene. Cytogenetic, comparative genomic hybridization (CGH), and LOH analyses of Wilms tumors showed gain or loss of specific chromosomes or chromosomal regions, indicating that *WT1*-wild-type tumors had more genomic alterations than *WT1*-mutant-type tumors (Nakadate et al., 1999; Hing et al., 2001; Ruteshouser et al., 2005). Furthermore, association of the long arm loss of chromosome 16 (16q−) with LOI of *IGF2* in Wilms tumor was recently reported (Mummert et al., 2005). However, 16q− was found in only a small portion of the tumors with LOI investigated, and no other cytogenetic abnormalities are known to be associated with LOI in the tumors. These studies indicate that Wilms tumor is a genetically heterogeneous disease, and further

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studies are needed to clarify the genetic/epigenetic and cytogenetic background of the tumor.

We evaluated the *WT1* and *IGF2* status and performed chromosome and/or CGH analysis of 43 Wilms tumors, on the basis of which we classified them into 4 genetic/epigenetic groups: *WT1* abnormality, LOH of *IGF2*, LOI of *IGF2*, and retention of imprinting (ROI) of *IGF2*. We analyzed the relationship between cytogenetic and genetic/epigenetic changes and found an association of LOI of *IGF2* with 11q− and +12 and possibly also with 16q−.

MATERIALS AND METHODS

Patient Samples

Tumor samples were available from 68 Japanese infants or children ranging in age from 2 months to 8 years who underwent surgery or biopsy between August 1984 and February 2003. These samples were selected on the basis of tissue availability and were not gathered consecutively. Of the 68 patients, 21 were registered in the Japan Wilms Tumor Group Study (JWITS). Samples of normal tissue were obtained from either the peripheral blood or normal renal tissue adjacent to the tumor from the same patients. Informed consent was obtained from the parents, and the study design was approved by the ethics committee of Saitama Cancer Center. The tumors were staged according to the National Wilms' Tumor Study group (NWTS) staging system, and most patients were treated according to NWTS protocols (d'Angio et al., 1989). None of the 68 patients had a family history of Wilms tumor. One patient (275) had Drash syndrome, and another patient (953) had bilateral tumors; the remaining patients had sporadic and unilateral tumors (Table 1).

Histological Examination

In all tumors, the diagnosis of Wilms tumor was made with routine hematoxylin- and eosin-stained pathology slides by local pathologists from each institution according to the classification proposed by the Japanese Pathological Society and/or the NWTS pathology panel (Beckwith et al., 1978; Japanese Pathological Society, 1988). Twenty-one cases that were registered at the JWITS were also reviewed by the pathology panel.

Cytogenetic, Fluorescence In Situ Hybridization, and CGH Studies

Chromosomes from tumor cells were studied by methods reported previously (Nakadate et al., 1999), and karyotypes were described according to the International System of Human Cytogenetic Nomenclature (ISCN, 1995). Fluorescence in situ hybridization (FISH) using Vysis probes [CEP 3 (chromosome 3 centromere), CEP 12 (chromosome 12 centromere), CBFB (16q22), and MLL (11q23); Downers Grove, IL) were carried out as described previously (Watanabe et al., 2002). CEP 12 was used to detect trisomy 12 and CEP 3 was used as a control because chromosome and CGH analyses detected 2 copies of chromosome 3 in almost all Wilms tumors, and the CBFB and MLL probes were used to detect 16q− and 11q−, respectively. Karyotypes of 11 of the 43 tumors described in Table 1 were reported previously (Nakadate et al., 1999).

CGH analysis was performed as described previously (Kumon et al., 2000). A chromosomal region was considered overrepresented or underrepresented if the average ratio profile was above 1.25 or below 0.75, respectively.

Analyses of WT1 Abnormalities and Allelic Loss on 11p and 11q

DNA preparation and digestion and Southern blot analysis using a *WT1* cDNA probe (WT33; Call et al., 1990), PCR–single-strand conformation polymorphism (SSCP) and subsequent direct-sequencing analysis, and allelic loss analysis on 11p and 11q were performed as described previously (Nakadate et al., 2001). Whether there was allelic loss on 11p and 11q was determined by PCR using microsatellite markers of D11S922, *TH*, *IGF2*, D11S932, *PAX6*, D11S903, D11S4100, *NCAM*, D11S1885, D11S29, and D11S1364 and using the restriction fragment length polymorphism (RFLP) sites of *WT1* (Tadokoro et al., 1993). The primer sequences used for PCR were obtained from the Genome Database (http://www.gdb.org). The results of the allelic loss analysis on 11p and 11q for 21 of the 43 tumors described in Tables 1 and 2 were reported previously (Nakadate et al., 2001).

The results of the study of promoter hypermethylation of *WT1* were reported previously (Satoh et al., 2003).

Analysis of *IGF2* Allelic Expression and Loss

The *ApaI*/*Ava*II polymorphism site in exon 9 was used to evaluate allelic expression of *IGF2*. PCR with genomic DNA from normal tissue and identification of heterozygous specimens after *Ava*II and *Hin*fI digestion were performed as described previously (Watanabe et al., 2002). RT-PCR products from the tumor RNA also were

TABLE 1. Clinical, Genetic, Karyotypic and CGH findings in 43 Wilms Tumors

Patients number	Age/Sex	Stage of disease	*WT1* Abnormality	Karyotype	CGH	CEP 12/CEP 3	CBFB
Tumors with *WT1* abnormalities and LOH or ROI of *IGF2* (n = 5)							
275*	1 y 0 m/F	I	Mutation in exon 8	48,XX,+3,+6	ND		
832*	9 m/F	II	Mutation in exon 2	45,XX,del(3)(p12p14),−7	ND		
949*	1 y 3 m/F	II	Promoter methylation	44,X,-X,dic r(1;11)(p3?q3?;q25?p1?),inv(9)(p11q12)c	ND		
2375	1 y 9 m/M	IV	Homozygous deletion	46,XY	N		
M289	5 y 4 m/F	II	Mutation in exon 7	ND	enh(18),dim(11p13–11q12, 19,22)		
Tumors with LOH of *IGF2* and no *WT1* abnormalities (n = 13)							
325*	1 y 6 m/M	I	None	47,XY,+8,del(14)(q22)	ND		
528*	4 y 1 m/F	II	None	56,XX,+5,+7,+7,+9,+10,+12,+13,+18,+19,+22	enh(1q,4p,7,8,9,10,12,13,18)	3/2	2
575	3 y 11 m/M	II	None	46,XY	enh(1q)		
871	1 y 4 m/F	I	None	NM	N	2/2	2
918*	4 y 6 m/M	III	None	45,X,-Y	ND		
1075	2 y 4 m//M	IV	None	NM	N		
1390	4 y 0 m/M	I	None	NM	enh(Yq)		
1570*	11 m/F	II	None	51,XX,+7,+8,+10,+12,+13,?del(16)(q22)	enh(7,12,13),dim(16q22–qter)	3/2	1
1658*	2 y 8 m/M	III	None	46,XY,der(16)t(1;16)(q21;q12)	ND		
1752	1 y 0 m/F	III	None	46,XX	N		
2488	3 m/F	I	None	46,XX	N		
M134	10 m/F	II	None	ND	enh(6q),dim(7p)		
M204	3 y 9 m/F	IV	None	ND	enh(8,9,20),dim(Y)		
Tumors with LOI of *IGF2* and no *WT1* abnormalities (n = 7)							
548	3 y 1 m/M	II	None	NM	enh(6,8,9,12)		
1206*	3 y 10 m/F	II	None	50,XX,+12,inc/11q- detected by FISH (MLL)	ND	3/2	2
1207	4 y 4 m/F	III	None	76–87 complex changes	enh(12),dim(9,10p,11q,16q,18p)		
1435*	6 y 1 m/F	III	None	53,XX,+12,inc	ND	3/2	2
1535*	3 y 8 m/F	II	None	46,XX,dup(1)(q21q25),der(11)t(1;11)(q21;q22),del(16)(q22)	enh(1q,4p15–pter),dim(11q13–qter,16q)		
M269	4 y 6 m/F	IV	None	ND	enh(7q,14q21–qter),dim(7p,X)		
M291	8 y 0 m/F	I	None	ND	enh(1q,6,9p,12,13,18q),dim(1p,11q,19)		

(Continued)

TABLE 1. Clinical, Genetic, Karyotypic and CGH findings in 43 Wilms Tumors (Continued)

Patients number	Age/Sex	Stage of disease	*WT1* Abnormality	Karyotype	CGH	CEP 12/CEP 3	CBFB
Tumors with ROI of *IGF2* and no *WT1* abnormalities (n = 18)							
884	2 m/M	III	None	46,XY	N	2/2	2
953	1 y 1 m/F	V	None	47,XX,add(2)(p25), del(7)(q11q22),+8	ND		
1371	5 m/F	IV	None	NM	N		
1420	6 m/F	Unknown	None	46,XX	N		
1879	7 m/M	I	None	46,XY	N		
2011	2 y 7 m/M	II	None	55,XY,+2,+6,+7,+8, +10,+del(12)(q23) +del(12)(q23),+13,+14	enh(1q,2,6,7q21–qter,8,10, 12pter–q23,13,15), dim(1p,18p)		
2385	1 y 4 m/F	IV	None	46,XX	N		
2677	4 y 4 m/F	II	None	46,XX	enh(2)		
2749	5 y 2 m/M	II	None	46,XY	dim(22)		
M126	2 y 5 m/F	III	None	ND	enh(2p14–pter,3q,6,7,8, 12,13,17)		
M175	1 y 9 m/F	I	None	ND	N		
M188	1 y 0 m/M	I	None	ND	N		
M196	1 y 5 m/F	III	None	ND	N		
M232	1 y 2 m/F	II	None	ND	N		
M233	5 y 3 m/F	IV	None	ND	enh(6,8)		
M238	6 m/F	I	None	ND	enh(7,8,10,12,13,17,18)		
M258	4 m/M	I	None	ND	N		
M290	2 y 1 m/M	II	None	ND	enh(1q,6,7,9,12),dim(18p,Y)		

*Karyotypes of these tumors were reported previously (Nakadate et al., 1999).
Abbreviations: NM, no mitotic cells; ND, not done; N, normal; 3/2, 3 copies of CEP 12 and 2 copies of CEP 3 detected by FISH; 2, 2 copies of CBFB detected by FISH.

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TABLE 2. Allelic Status of 11p and 11q and *IGF2* Imprinting Status in *WT1*, LOH of *IGF2*, and LOI of *IGF2* Wilms Tumor Groups

	11p15					11p13		11p11	11q21–22	11q23					
	S922	*IGF2*	*IGF2-LOI*	*TH01*	S932	*PAX6*	*WT1*	S903	S4100	*NCAM*	S1855	S29	S1364	*WT1* abnormality[a]	11q-detected by CGH/ cytogenetics
Tumors with *WT1* abnormalities and LOH, LOI, or ROI of *IGF2* (n = 5)															
275	—	●	—	●	●	—	●	—	—	○	—	○	—	Mutation in exon 8	Not detected
832	—	—	—	●	●	●	●	—	○	○	—	—	○	Mutation in exon 2	Not detected
949	—	—	—	●	—	—	●	●	●	—	—	—	●	Promoter methylation	Not detected
M289	—	○	□	○	○	○	●	●	—	—	○	○	○	Mutation in exon 7	Not detected
2375	○	○	□	○	○	—	▲	—	○	○	—	—	○	Homozygous deletion	Not detected
Tumors with LOH of *IGF* and no *WT1* abnormalities (n = 13)															
325	●	—	—	—	—	—	●	—	○	○	—	○	○	None	Not detected
528	—	●	—	●	●	●	—	—	●	—	●	—	●	None	Not detected
575	●	●	—	●	●	—	○	—	○	○	—	—	○	None	Not detected
871	●	—	—	●	—	●	—	—	—	○	○	○	○	None	Not detected
918	●	—	—	—	—	○	○	○	—	—	○	○	○	None	Not detected
1075	—	—	—	●	●	—	○	○	○	○	○	—	○	None	Not detected
1390	—	—	—	●	—	—	●	○	○	○	○	—	—	None	Not detected
1570	—	●	—	—	●	●	—	—	—	●	●	●	—	None	Not detected
1658	—	—	—	●	—	—	—	—	—	—	○	○	—	None	Not detected
1752	●	—	—	●	●	—	●	●	—	—	—	○	○	None	Not detected
2488	●	—	—	—	●	●	●	—	○	—	○	○	○	None	Not detected
M134	—	●	—	—	●	●	●	●	○	○	○	○	—	None	Not detected
M204	—	—	—	—	●	●	●	—	—	●	—	—	○	None	Not detected
Tumors with LOI of *IGF2* and no *WT1* abnormalities (n = 7)															
548	—	○	■	○	○	—	○	—	○	—	—	○	○	None	Not detected
1206	—	○	■	○	○	○	○	○	○	—	—	●	●	None	Detected
1207	—	○	■	○	—	—	—	○	—	—	—	—	●	None	Detected
1435	—	○	■	—	—	○	○	—	○	○	—	○	○	None	Not detected
1535	○	○	■	○	—	—	○	—	—	●	●	●	—	None	Detected
M269	○	○	■	○	○	○	○	○	○	—	—	○	○	None	Not detected
M291	○	—	■	○	—	○	○	—	●	—	●	●	●	None	Detected

[a]Details of *WT1* abnormality are described in the text.
● Loss of heterozygosity; ○ Retention of heterozygosity; — Not informative; ■ Loss of *IGF2* imprinting; □ Retention of *IGF2* imprinting; ▲ Homozygous *WT1* deletion.

digested with *Ava*II and *Hinf*I, and allelic expression of *IGF2* was determined.

Statistical Analysis

The significance of differences in various clinical and cytogenetic aspects of the disease among the 4 genetic/epigenetic groups of tumors was determined by the chi-square or Fisher's exact tests. Differences in the mean age of the patients and in the average number of chromosome changes between any 2 of the 4 groups were examined with Welch's t test.

RESULTS

Allelic Loss on 11p and 11q

Allelic loss on 11p and 11q was analyzed in Wilms tumor samples from 68 patients. Informative 11p15 loci were found in normal tissue from 64 of the patients; the 11p15 loci in the tissue from the other 4 patients were uninformative. Of the 64 informative tumors, 16 showed LOH. Of the 48 tumors without LOH, 27 were informative for the *Apa*I/*Ava*II polymorphism site of the *IGF2* gene. Thus, 43 tumor samples were the subject of the present study.

Three tumors (949, 528, and 1570) showed LOH for the entire chromosome 11; 1 tumor (M204) showed LOH on 11p15–11q23, retaining heterozygosity in the more distal 11q locus; 3 tumors (575, 918, and 1075) showed LOH limited to the 11p15 region; and 9 (275, 832, 325, 871, 1390, 1658, 1752, 2488, and M134) showed LOH limited to the 11p15–11p13 region (Table 2). Of the 27 tumors without LOH on 11p15, 1 (M289) showed

LOH limited to 11p13–11p11, and 4 (C1206, C1207, C1535, and M291) showed LOH on 11q (Table 2).

WT1 Abnormalities

Of the 9 tumors with LOH limited to the 11p15–11p13 region, 2 showed a *WT1* mutation; one (275) had a missense mutation in exon 8 (G to A conversion in nucleotide 1064; Haber et al., 1991), and the other (832) had a nonsense mutation (C to T conversion in nucleotide 550) in exon 2 (Table 2). Another tumor (C949) was found to have *WT1* promoter methylation, which was examined in 21 of the 43 tumors, of which only 1 showed the methylation (Satoh et al., 2003). This tumor had a ring chromosome containing chromosomes 1 and 11. Because the incidence of promoter methylation was quite low, and no other tumors showed a ring chromosome containing chromosome 11 and LOH for the entire chromosome 11, the other 22 tumors whose *WT1* promoter methylation status was not examined were assumed to be unmethylated.

Of 27 tumors without LOH on 11p15, 1 (M289) with LOH limited to the 11p13–11p11 region had a missense mutation in exon 7 (G to T conversion in nucleotide 895), and another (C2375) with retention of heterozygosity (ROH) for the entire chromosome 11 had homozygous deletion of the 6.6-kb fragment of *WT1*, detected by Southern blotting with a *WT1* cDNA probe and *Eco*RI digestion (Call et al., 1990; Table 2).

LOI of *IGF2*

Of the 27 tumors with ROH in 11p15 and the informative *Apa*I/*Ava*II polymorphism site of *IGF2*, 7 showed LOI of *IGF2* (Tables 1 and 2, Fig. 1). Of the 20 ROI tumors, 2 (M289 and C2375) showed *WT1* abnormalities as described before.

Four Groups of Tumors Classified by *WT1* and *IGF2* Status

We classified 43 Wilms tumors into 4 groups on the basis of major genetic abnormalities: *WT1* abnormality, LOH of *IGF2*, LOI of *IGF2*, and tumors without *WT1* or *IGF2* abnormalities. Three tumors with a *WT1* abnormality and LOH on 11p15–11p13 were included in the *WT1* group because *WT1* abnormalities are believed to have a stronger impact on tumorigenicity than LOH of *IGF2*. Thus, of the 43 tumors, 5 were classified into the *WT1* group, 13 into the LOH group, 7 into the LOI group, and 18 into the ROI group (Table 1).

CGH patterns and/or karyotypes were available for all 43 tumors (Table 1). Four tumors (528, 1206, 1435, and 1570) with a hyperdiploid karyotype (≥50 chromosomes) and trisomy 12 with or without other changes were also studied by FISH using the CEP 3, CEP 12, and CBFB probes. All 4 tumors were shown to have trisomy 12, and 1 was shown to have 16q−. One tumor (1206) was shown to have 11q− using FISH with the *MLL* probe.

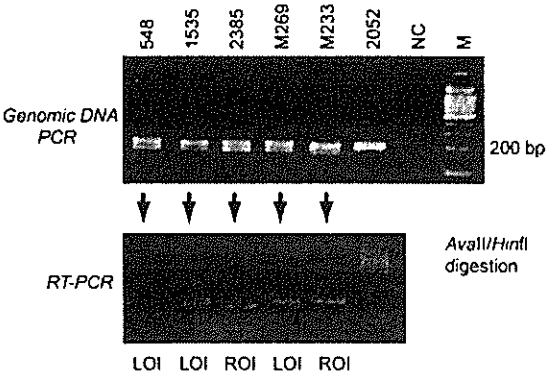

Figure 1. Electrophoretic patterns of products of genomic DNA PCR or reverse-transcription PCR after *Ava*II and *Hin*fI digestion. Normal tissue from samples 548, 1535, 2385, M269, and M233 was observed to have heterozygous *IGF2* alleles, and normal tissue from sample 2052 was observed to have homozygous *IGF2* alleles, in upper lanes; loss of imprinting was found in tumor tissue from samples 548, 1535, and M269 and retention of imprinting in tumor tissue from samples 2385 and M233, in lower lanes [NC, negative control (H_2O); M, size marker].

Clinical Characteristics of Patients in Each Tumor Group

The mean age of the patients was higher in the LOI group than in the *WT1* ($P = 0.03$), the LOH ($P = 0.01$), the ROI ($P < 0.01$), or the *WT1* + LOH + ROI ($P < 0.01$) groups (Table 3). There were no differences in stage distribution among the 4 groups. The tumors of 42 patients were classified as having a favorable histology, and the tumor of 1 patient (1390) was classified as having unfavorable histology (the diffuse anaplasia type). Of the 43 patients, 41 were alive with no evidence of disease at the last follow-up (November 30, 2004). Two patients had died: the patient who had the diffuse anaplasia–type tumor died of the disease, and the patient in the *WT1* group who had Drash syndrome (275) died of renal failure.

Association of Chromosome Abnormalities with *IGF2* and *WT1* Status

Ten chromosome/CGH abnormalities were seen in 4 or more tumors (Table 3). Loss limited to 11q was more frequent in the LOI group than in the *WT1* ($P = 0.08$), the LOH ($P < 0.01$), the ROI ($P < 0.01$), or the *WT1* + LOH + ROI ($P < 0.01$)

TABLE 3. Relationship between Cytogenetic Abnormalities with 4 Wilms Tumor Groups Classified by *WT1* or *IGF2* Status

Tumors classified by *WT1* and *IFG2* status	Number of tumors	Mean age of patients in months (range)	Mean number of cytogenetic changes	+1q	+6	+7/+7q	7p-	+8	+10	11q-[a]	+12[b]	+13	16q-[c]
A. Tumors with *WT1* abnormalities and LOH or ROI of *IGF2*	5	24.2 (9–64)	0.4	0	1	0	1	0	0	0	0	0	0
B. Tumors with LOH of *IGF2* and no *WT1* abnormalities	13	28.7 (3–54)	1.5	3	1	2	1	4	2	0	2	2	2
C. Tumors with LOI of *IGF2* and no *WT1* abnormalities	7	57.4 (37–96)	2.7	2	2	1	1	1	0	4	5	1	2
D. Tumors with ROI of *IGF2* and no *WT1* abnormalities	18	23.2 (2–63)	1.3	2	4	4	0	6	2	0	4	3	0

Mean age: C versus A, $P = 0.03$; C versus B, $P = 0.01$; C versus D, $P < 0.01$; C versus A+B+D, $P < 0.01$.
Mean number of cytogenetic changes: A versus B, $P = 0.12$; A versus C, $P < 0.01$; A versus D, $P = 0.13$; A versus B+C+D, $P < 0.01$.
[a]11q-: C versus A, $P = 0.08$; C versus B, $P < 0.01$; C versus D, $P < 0.01$; C versus A+B+D, $P < 0.01$; C versus A (2 tumors with ROI)+D, $P < 0.01$.
[b]+12: C versus A, $P = 0.03$; C versus B, $P = 0.02$; C versus D, $P = 0.06$; C versus A+B+D, $P < 0.01$; C versus A (2 tumors with ROI)+D, $P = 0.02$.
[c]16q-: C versus A, $P = 0.46$; C versus B, $P = 0.6$; C versus D, $P = 0.06$; C versus A+B+D, $P = 0.12$; C versus A (2 tumors with ROI)+D, $P = 0.06$.

groups. When we added 4 tumors with LOH in the entire chromosome 11 or in the 11p15–11q23 region to the 11q− category, 11q− was still more frequent in the LOI group than in the *WT1*+LOH+ROI group ($P = 0.02$).

Trisomy 12 was more frequent in the LOI group than in the *WT1* ($P = 0.03$), LOH ($P = 0.02$), ROI ($P = 0.06$), or LOH+ROI+*WT1* ($P < 0.01$) groups. Loss of 16q was found only in the LOI or the LOH group, but there was no significant difference among the 4 groups, or between the LOI and the *WT1*+LOH+ROI groups ($P = 0.12$). Mummert et al. (2005) excluded tumors with LOH on 11p15 in a correlation analysis of 16q− and LOI of *IGF2* because LOI of the maternal *IGF2* allele prior to its deletion could not be ascertained. When we excluded 16 tumors with LOH on 11p15, 16q− tended to be more frequent in the LOI group than in the *WT1* group with the ROI of *IGF2* + ROI group ($P = 0.06$). No other associations between chromosome abnormalities with any of the 4 groups were found (Fig. 2).

For the 10 chromosome/CGH abnormalities observed in 4 or more tumors, the mean number per tumor was lower in the *WT1* group (0.4/tumor) than in the LOH (1.5/tumor; $P = 0.12$), LOI (2.7/tumor; $P < 0.01$), ROI (1.3/tumor; $P = 0.13$), or LOH+LOI+ROI (1.7/tumor; $P < 0.01$) groups (Table 3).

DISCUSSION

Wilms tumor is a heterogeneous disease showing various genetic/epigenetic abnormalities, including mutations/deletions of the *WT1* gene, LOH or LOI of the *IGF2* gene, and *CTNNB1* mutations frequently associated with *WT1* abnormalities (Ogawa et al., 1993; Rainier et al., 1993; Koesters et al., 1999; Mati et al., 2000; Ravenel et al., 2001). In addition, we previously reported that hyperdiploid tumors, usually including trisomy 12, might be a unique subgroup of tumors with no *WT1* abnormalities (Nakadate et al., 1999). Cytogenetic, CGH, and LOH studies have found recurrent abnormalities, including gains of 1q, 2, 6, 7, 8, 10, 12, 13, and 18 and losses of 1p, 7p, 9q, 11p, 11q, 16q, and 22q (Nakadate et al., 1999; Hing et al., 2001; Ruteshouser et al., 2005). None of the previous studies simultaneously examined the status of *WT1*, LOH or LOI of *IGF2*, LOH on 11p and 11q, and all chromosome/CGH patterns. The present study showed *WT1* abnormalities, LOH of *IGF2*, LOI of *IGF2*, and ROI of *IGF2* in 12%, 30%, 16%, and 42%, respectively, of 43 Wilms tumors.

Recently, Mummert et al. (2005) reported that Wilms tumors with 16q− had expression of *CTCF* half that of expression in tumors with normal chromosomes 16 and that LOI of *IGF2* was associated with loss of 16q. The *CTCF* gene, at 16q22, codes for an insulator protein. According to Mummert et al. (2005), when less CTCF was available to bind the differentially methylated region (DMR) upstream of *H19*, access of maternal *IGF2* to an enhancer downstream of *H19* might occur. The present study confirmed that tumors with 16q− showed either LOI or LOH of *IGF2* (Yeh et al., 2002; Mummert et al., 2005) and provided support for the association of 16q− with LOI of *IGF2*. Furthermore, the present study disclosed that 11q− and +12 were more frequent in tumors with LOI than in those with LOH, ROI, or *WT1* abnormalities.

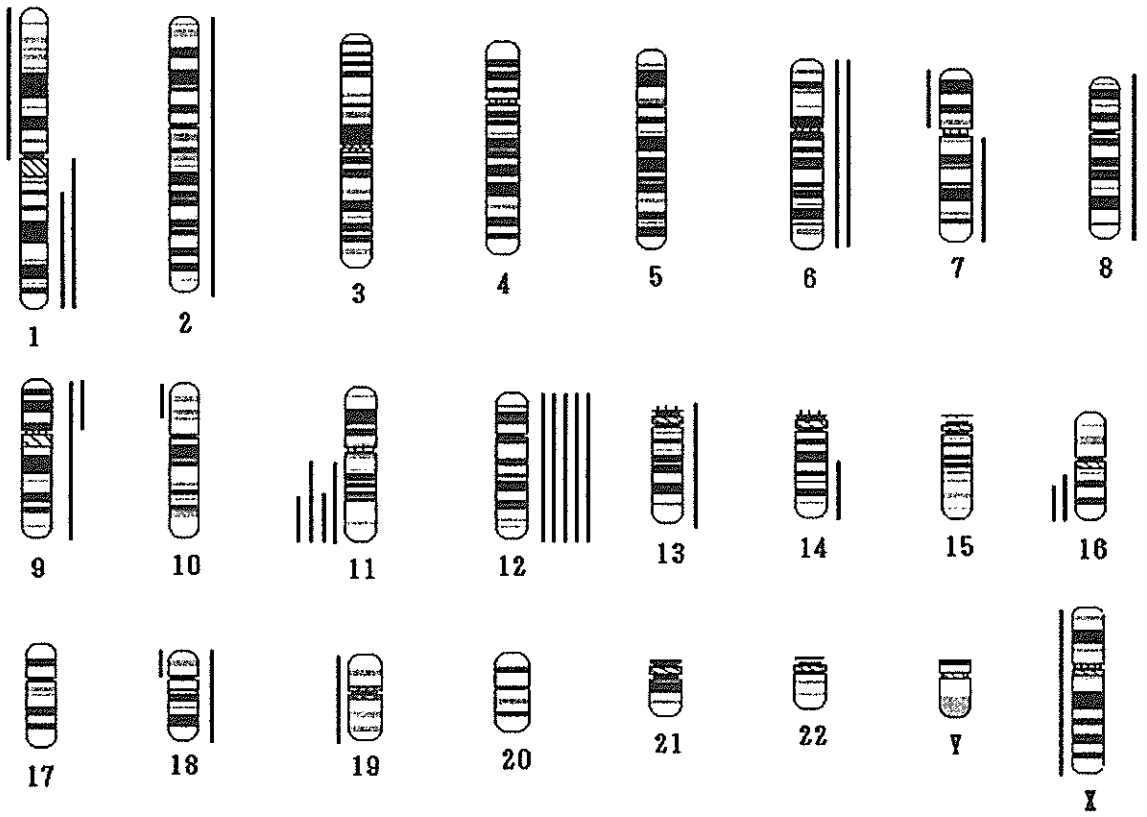

Figure 2. Summary of chromosome changes in the LOI group detected by CGH, chromosome, FISH, and/or LOH analyses. Gains and losses are shown on the right and left sides, respectively.

Overexpression of *IGF2* can be caused by LOI or by duplication of the paternal chromosome 11 with loss of the maternal chromosome 11 (LOH). LOI or LOH of *IGF2* has been detected in various embryonal tumors, including Wilms tumor, rhabdomyosarcoma, and hepatoblastoma (Ogawa et al., 1993; Rainier et al., 1993, 1995; Zhan et al., 1994). More recently, microdeletion of the maternal *H19* DMR was reported in a large family of people with Beckwith–Wiedemann syndrome (Prawitt et al., 2005). Although LOI of *IGF2* was found in fibroblasts from all 4 individuals with the microdeletion, 3 with a second genetic lesion (duplication of the microdeleted maternal *IGF2* locus), but not the one without it, developed Beckwith–Wiedemann syndrome and Wilms tumor. These findings suggest that LOI of *IGF2* or duplication of the paternal *IGF2* may be one of several genetic and epigenetic events that promote tumor cell proliferation.

The present study found an association of 11q– with LOI of *IGF2*. Very recently, Yuan et al. (2005) studied LOI of *IGF2* by assessing DNA methylation of the *H19* DMR and LOH by single-nucleotide polymorphism (SNP) chips in 58 sporadic Wilms tumors, 22 of which showed LOI. Partial loss of 11q and loss of whole chromosome 11 were found in 6 and 0, respectively, of the 22 LOI tumors, and in 1 and 13, respectively, of the 36 non-LOI tumors. They stated that 11q– was not associated with LOI. When we added 4 tumors with LOH for the entire chromosome 11 or the 11p and 11q regions into the 11q– category in the present series, 11q– was still more frequent in the LOI group than in the *WT1* + LOH + ROI group. Whole loss of chromosome 11 may play a role in loss of the wild-type *WT1* allele or in loss of the maternal *IGF2* allele, and 11q– may be a bystander in tumors with whole loss of chromosome 11 and *WT1* mutation or duplication of the paternal *IGF2* (LOH). When the 13 tumors with loss of the entire chromosome 11 from the series reported by Yuan et al. (2005) were excluded, partial loss of 11q was more frequent in the LOI tumors (6 of 22 tumors) than in the non-LOI tumors (1 of 23 tumors), $P < 0.01$, Fisher's exact

test. Thus, the present study and that of Yuan et al. (2005) lead to the same conclusion: chromosomal loss limited to 11q is associated with LOI of *IGF2* in Wilms tumor.

It has been hypothesized that 11q harbors a tumor-suppressor gene involved in the development of Wilms tumor (Radice et al., 1995; Nakadate et al., 2001). The association between 11q− and LOI of *IGF2* found in the present study suggests that Wilms tumors with overexpression of *IGF2* require deletion/mutation of the putative 11q gene in order to develop to full-blown tumors. As we have shown (Tables 1 and 2, Fig. 2), the present CGH and cytogenetic study detected physical loss of 11q DNA, rather than mitotic recombination, in the 4 tumors with LOI and 11q LOH. The gene on 11q may code for one of the proteins that constitute a CTCF insulator complex, and mutation, deletion, or haploinsufficiency of the gene may cause insulator abnormalities that might lead to LOI of *IGF2* (Ohlsson et al., 2001).

The present study also found an association between trisomy 12 and LOI of *IGF2*. We previously proposed that hyperdiploid tumors (≥50 chromosomes) make up a unique subgroup of Wilms tumors characterized by the absence of *WT1* abnormalities and nonrandom gains of chromosomes, usually including trisomy 12 (Nakadate et al., 1999). The present study added another characteristic, namely, the tendency to show LOI of *IGF2*, to the list of characteristics of hyperdiploid tumors. *CCND2* and *CDK4*, which are growth-promoting genes on chromosome 12, are overexpressed in Wilms tumors (Faussillon et al., 2005), and it is speculated that tumors with LOI of *IGF2* also need trisomy 12 in order to proliferate in an accelerated manner.

Ravenel et al. (2001) reported that patients who had Wilms tumors with LOI of *IGF2* were older than those who had tumors with normal imprinting and that the tumors with LOI were more likely to be of a pathological subtype associated with a later stage of renal development. The present study confirmed that patients with tumors with LOI were older than those who had tumors of other subtypes. Chromosome changes were most frequent in the LOI group and least frequent in the *WT1* group (Table 3). We suggest from the findings described above that tumors with LOI need far more genetic events to develop into full-blown tumors than do those with certain genetic types of tumors; it will take time to accumulate the genetic and epigenetic events that might explain why patients with LOI of *IGF2* are older.

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Genetic and epigenetic alterations on the short arm of chromosome 11 are involved in a majority of sporadic Wilms' tumours

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Wilms' tumour is one of the most common solid tumours of childhood. 11p13 (*WT1* locus) and 11p15.5 (*WT2* locus) are known to have genetic or epigenetic aberrations in these tumours. In Wilms' tumours, mutation of the *Wilms tumour 1* (*WT1*) gene at the *WT1* locus has been reported, and the *WT2* locus, comprising the two independent imprinted domains *IGF2/H19* and *KIP2/LIT1*, can undergo maternal deletion or alterations associated with imprinting. Although these alterations have been identified in many studies, it is still not clear how frequently combined genetic and epigenetic alterations of these loci are involved in Wilms' tumours or how these alterations occur. To answer both questions, we performed genetic and epigenetic analyses of these loci, together with an additional gene, *CTNNB1*, in 35 sporadic Wilms' tumours. Loss of heterozygosity of 11p15.5 and loss of imprinting of *IGF2* were the most frequent genetic (29%) and epigenetic (40%) alterations in Wilms' tumours, respectively. In total, 83% of the tumours had at least one alteration at 11p15.5 and/or 11p13. One-third of the tumours had alterations at multiple loci. Our results suggest that chromosome 11p is not only genetically but also epigenetically critical for the majority of Wilms' tumours.
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Wilms' tumour, also known as nephroblastoma, is one of the most common solid tumours of childhood, accounting for approximately 6% of all childhood malignancies. Chromosomal region 11p13 was first identified as a Wilms' tumour locus, *WT1*, because the region was found to be deleted in Wilms' tumours (Kaneko *et al*, 1981; Huff, 1998; Dome and Coppes, 2002, OMIM 194070). The *Wilms tumour 1* (*WT1*) gene, isolated from the *WT1* locus, was the first causative gene for Wilms' tumour (Call *et al*, 1990; Gessler *et al*, 1990). However, *WT1* aberrations, such as deletions and point mutations, are observed in only approximately 10–20% of Wilms' tumours (Huff, 1998; Nakadate *et al*, 2001). The small number of *WT1* mutations in Wilms' tumours suggests that *WT1* can be inactivated by alterations that would not be detected by mutational analysis (Huff, 1998). On the other hand, although *WT1* mutation is not frequent, *WT1* mutation and *CTNNB1* (*β-catenin*) mutation at 3p21 are significantly correlated with Wilms' tumours (Maiti *et al*, 2000).

Loss of heterozygosity (LOH) of 11p15.5, which is known as the *WT2* locus (OMIM 194071), is observed in Wilms' tumours. LOH occurs on the maternal chromosome, suggesting the involvement of genomic imprinting in Wilms' tumorigenesis. This imprinted region is well characterised, and is divided into two imprinted domains, *IGF2/H19* and *KIP2/LIT1* (Feinberg, 2000). It has been reported that *IGF2* and *H19* within the *IGF2/H19* domain are expressed abnormally in Wilms' tumours. *IGF2* encodes an embryonal growth factor and is transcribed exclusively from the paternal allele (Reik and Murrell, 2000), and *H19* is a noncoding RNA with reciprocal transcription from the maternal allele. In Wilms' tumours, abnormally high levels of *IGF2* mRNA and loss of imprinting (LOI) of *IGF2*, allowing both paternal and maternal alleles to be transcribed, have been observed (Reeve *et al*, 1985; Ogawa *et al*, 1993; Rainier *et al*, 1993). LOI of *IGF2* is always accompanied by *H19* biallelic hypermethylation, leading to inactivation of *H19* (in the normal situation, the region upstream of *H19* is methylated only on the paternal allele) (Moulton *et al*, 1994; Steenman *et al*, 1994). Demethylation of *DMR-LIT1*, an imprinting control region (ICR) of the *KIP2/LIT1* domain, occurs in half of all patients with Beckwith–Wiedemann syndrome (BWS) (OMIM 130650), which predisposes patients to embryonal tumours, and in a variety of adult tumours. The $p57^{KIP2}$ (*KIP2*)/*CDKN1C* gene within the *KIP2/LIT1* domain, which is expressed predominantly from the maternal allele, encodes a cyclin-dependent kinase inhibitor and is a putative tumour suppressor. In several adult tumours, *KIP2* expression is epigenetically reduced (Shin *et al*, 2000; Kikuchi *et al*, 2002; Li *et al*, 2002; Soejima *et al*, 2004). However, *KIP2* expression has been found to be reduced in Wilms' tumours in some studies, but not in others (Chung *et al*,

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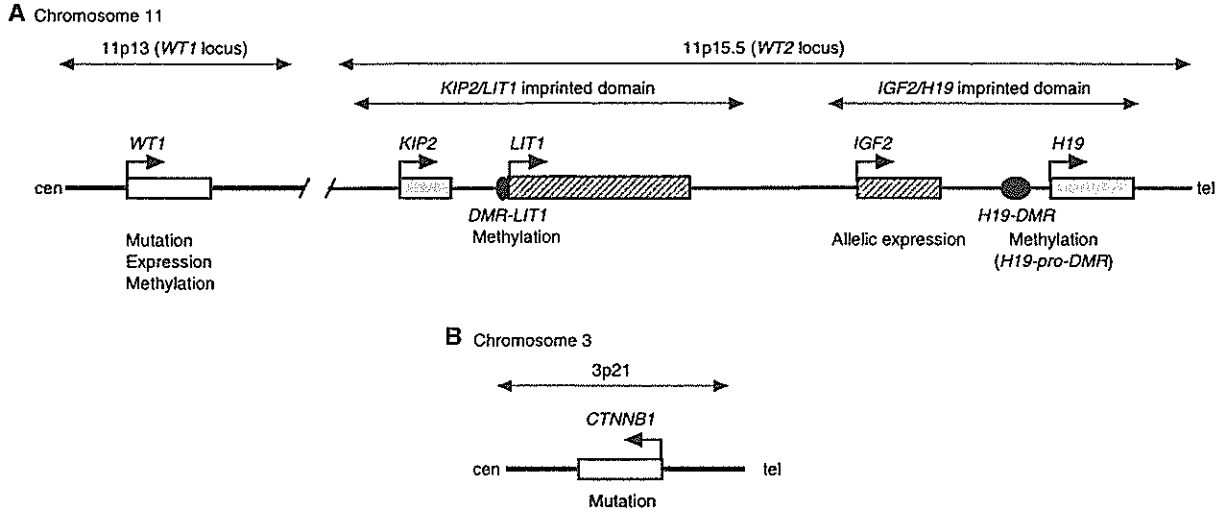

Figure 1 Maps of regions analysed in this study. (**A**) *WT1* locus and *WT2* locus on the short arm of chromosome 11. Representative genes are shown. Broken arrows indicate transcriptional direction. Grey boxes and shaded boxes indicate maternal and paternal expression, respectively. *DMR-LIT1* and *H19-DMR* are the ICRs for each domain, respectively. *DMR-LIT1* is differentially methylated on the maternal allele. The *H19-DMR* and *H19* promoter are differentially methylated on the paternal allele. The items examined in this study are shown below each gene or DMR. (**B**) *CTNNB1* locus. Maps are not to scale.

1996; Hatada *et al*, 1996; Thompson *et al*, 1996; O'Keefe *et al*, 1997; Taniguchi *et al*, 1997; Soejima *et al*, 1998).

Although several genes involved in Wilms' tumour have been identified, as described above, the alteration frequencies of these genes (loci) and how many loci are altered in the tumour are still unknown. To investigate this, we comprehensively investigated genetic and epigenetic alterations of three loci - *WT1* (11p13), *WT2* (11p15.5), and *CTNNB1* (3p21) — in 35 sporadic Wilms' tumours (Figure 1). Our data indicate that genetic and/or epigenetic alterations of genes at these loci, especially *WT1* and *WT2*, is involved in the majority of Wilms' tumours, and that alterations of multiple loci occur in one-third of tumours. These findings suggest that genetic and epigenetic alterations on the short arm of chromosome 11 play an important role in Wilms' tumorigenesis.

MATERIALS AND METHODS

DNA and RNA

In all, 35 tissue samples from sporadic Wilms' tumours and five tissue samples from mid-gestational fetal kidneys were obtained from Saitama Cancer Center Hospital (Saitama, Japan) and the fetal tissue bank at the University of Washington, (WA, USA), respectively. Genomic DNA and total RNA were extracted with a QIAamp DNA mini kit (Qiagen, Hilden, Germany) and Isogen (Nippon Gene, Tokyo, Japan), respectively.

Mutation and LOH analyses

Genetic analyses of *WT1* were carried out as previously described (Nakadate *et al*, 1999, 2001). Briefly, *WT1* loci were screened for mutations by single-strand conformation polymorphism (SSCP) analysis of all exons and splice-donor/acceptor sites. When an aberrant band was identified by SSCP, the band was excised and sequenced. Loss of heterozygosity was also analysed using polymorphic DNA markers as follows to compare tumour tissue with adjacent normal tissue or peripheral blood: *D11S16*, *D11S325*, *PAX6*, *D11S324*, *WT1*, and *CAT* for 11p13; and *D11S12*, *D11S922*, *D11S932*, *IGF2*, *INS*, and *TH* for 11p15.5. Mutations in exon 3 of the *CTNNB1* gene were investigated by PCR-directed sequencing as previously described (Satoh *et al*, 2003).

Quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA (500 ng) was treated with RNase-free DNase I (Roche, Basel, Switzerland) and reverse-transcribed with ReverTra Ace reverse transcriptase (Toyobo, Japan) and random primers (TaKaRa, Japan). Quantitative real-time RT-PCR was performed with the LightCycler™ system (Roche) according to the manufacturer's protocol. The expression of *WT1* was normalised with that of *β-actin*, as previously described (Satoh *et al*, 2003). The average *WT1* expression of four mid-gestational fetal kidneys was employed as a standard. All experiments were performed in triplicate.

Allele-specific expression of *IGF2*

Genotyping of *IGF2* was performed by PCR-restriction fragment length polymorphism (RFLP) using a polymorphic *Hae*III (*Apa*I or *Ava*II) site in exon 9, as previously described (Soejima and Yun, 1998). To eliminate genomic DNA contamination, the RNA-specific product (1120 bp) was amplified by using an exon connection primer pair (5′-TCCTGGAGACGTACTGTGCTA-3′ and 5′-GGTCGTGCCAATTACATTTCA-3′). To further eliminate contaminating DNA, the RNA-specific product was excised from 1% agarose gel after electrophoresis and purified. Then, the purified product was subjected to nested PCR and RFLP analysis with *Hae*III (Yun *et al*, 1999).

Methylation analyses

Combined bisulphite restriction analyses (COBRA) using the hot-stop method were employed to determine the extent of methylation at the differentially methylated region (DMR) of the *H19* promoter (*H19-pro-DMR*), *DMR-LIT1*, and *WT1* promoter. Although an ICR of the *IGF2/H19* domain exists between 2 and 5 kb upstream of the *H19* gene, we analysed *H19-pro-DMR* because *IGF2* LOI uniformly correlates with hypermethylation of *H19-pro-DMR* (Moulton *et al*, 1994; Steenman *et al*, 1994). The primer pairs and restriction endonucleases used were as

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