J. Biochem. 128, 847–853 (2000)

A Novel Imprinted Gene, *KCNQ1DN*, within the WT2 Critical Region of Human Chromosome 11p15.5 and Its Reduced Expression in Wilms' Tumors[1]

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Received July 31, 2000; accepted September 11, 2000

WT2 is defined by a maternal-specific loss of heterozygosity on human chromosome 11p15.5 in Wilms' and other embryonal tumors. Therefore, the imprinted genes in this region are candidates for involvement in Wilms' tumorigenesis. We now report a novel imprinted gene, *KCNQ1DN* (*KCNQ1* downstream neighbor). This gene is located between *p57*^{KIP2} and *KvLQT1* (*KCNQ1*) of 11p15.5 within the WT2 critical region. *KCNQ1DN* is imprinted and expressed from the maternal allele. We examined the expression of *KCNQ1DN* in Wilms' tumors. Seven of eighteen (39%) samples showed no expression. In contrast, other maternal imprinted genes in this region, including *p57*^{KIP2}, *IMPT1*, and *IPL* exhibited almost normal expression in these samples, although some samples expressed *IGF2* biallelically. These results suggest that *KCNQ1DN* existing far from the *H19/IGF2* region may play some role in Wilms' tumorigenesis along with *IGF2*.

Key words: CpG island, imprinted genes, genomic imprinting, *KCNQ1DN*, Wilms' tumors.

Genomic imprinting is an epigenetic modification that can lead to parental-allele-specific expression of genes, which ensures the functional inequality of paternal and maternal genomes in somatic cells (*1*, *2*). Many imprinted genes are clustered and constitute a relatively large imprinted chromosomal domain. The human chromosome 11p15.5 is a well studied imprinted region, since it harbors multiple imprinted genes. Furthermore, mutations or altered imprinting of these imprinted genes are associated with human diseases. These include: (i) Beckwith-Wiedemann syndrome (BWS, OMIM130650), which is an autosomal dominant disorder, characterized by prenatal overgrowth and predisposition to tumors (*3–10*); and (ii) tumors, including embryonal tumors such as Wilms' tumor, hepatoblastoma and rhabdomyosarcoma, as well as a variety of adult tumors (*11–15*). Wilms' tumor 2 (WT2), is defined by frequent loss of heterozygosity (LOH) of 11p15.5 in those tumors. The LOH involving the maternal allele (*16*) suggests that the imprinted genes in this region are involved in these tumors (see Fig. 1A).

At least ten imprinted genes in 11p15.5 have been identified and characterized, including four paternally (*IGF2*, *LIT1*, *PEG8*/*IGF2AS*, and *MTR1*) and six maternally (*IPL*, *IMPT1*, *p57*^{KIP2}, *KvLQT1*, *ASCL1*, and *H19*) transcribed genes (*17–23*). In principle, all of these genes are candidates for involvement in Wilms' tumorigenesis. Several of them with growth-related function have been thoroughly investigated. *IGF2*, insulin-like growth factor II gene, which is paternally transcribed, showed loss of imprinting (LOI) in 70% of Wilms' tumors and about half of all adult cancers (*24*, *25*). Transgenic mice with deregulated *IGF2* expression show some features of BWS, but neither exomphalos nor a predisposition to tumors (*26*), *H19* is maternally transcribed and codes an RNA without an open reading frame. It can be growth-suppressive in some cell types (*27*, *28*) and shows epigenetic biallelic silencing in some Wilms' tumors with LOI of *IGF2* (*29*), but no consistent imprinting defects nor mutations in *H19* have been described in Wilms' tumors, *p57*^{KIP2} is a cyclin-dependent kinase inhibitor and is preferentially transcribed from the maternal allele (*30–32*). This gene shows mutations in 5–17% of BWS patients (*7–10*) and reduced expression in some Wilms' tumors (*32–34*). Targeted disruption of *p57*^{KIP2} in mice exhibits some aspects of BWS resulting in abdominal muscle defects and kidney dysplasias, but does not show any features of Wilms' and other embryonal tumors (*35*). Furthermore, no mutation in *p57*^{KIP2} has been reported in Wilms' tumors, *IMPT1* (also known as *ITM*, *ORCTL2*, and *TSSC5*) is imprinted and expressed from the maternal allele in embryonal tissues. Mutations of this gene have

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[1] This work was supported by grants from the Ministry of Health and Welfare (Research on Human Genome and Therapy), and the Ministry of Education, Science, Sports and Culture of Japan, and the Uehara Memorial Foundation. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB039920.

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been found in some patients with Wilms' tumors (*36*).

Taken together, the above evidence for *IGF2*, *H19*, *p57*^{KIP2}, and *IMPT1* can account for some of Wilms' and other tumors. In addition, functional assays using microcell-mediated chromosome transfer identified a region harboring at least one tumor suppressor gene in 11p15.5 (*37*), suggesting that other associated genes remain to be found in this region. Here, we report that a novel imprinted gene, *KCNQ1DN*, which is located between *p57*^{KIP2} and *KvLQT1* within the WT2 critical region on 11p15.5, is expressed from maternal allele and shows reduced expression in Wilms' tumors.

MATERIALS AND METHODS

Sequence Analysis and Screening of Human Testis cDNA Library—EST database searches were performed using the BLAST programs on the NCBI server (http://www.ncbi.nlm.nih.gov/). The IMAGE clone 1422939 was obtained from the American Type Culture Collection. The clone was amplified as indicated by the provider. Sequencing was performed using M13 and M13 reverse primers on an ABI 377 sequencer (Applied Biosystems, Germany). Human testis cDNA library (Clontech, USA) was screened with the insert of IMAGE clone 1422939 as a probe.

Isolation of DNA and RNA from Tissues—Normal fetal tissues were obtained from the fetal tissues bank at the University of Washington. DNA was extracted by the standard phenol-chloroform method. RNA was extracted using Isogen (Nippongene, Tokyo) according to the manufacturer's protocol.

Expression Analysis—Total RNAs of multiple human adult tissues were obtained from OriGene Technologies (USA). To obtain the expression profile of *KCNQ1DN*, first strand cDNA synthesis was carried out with an oligo(dT)$_{15}$ primer and AMV reverse transcriptase (Takara Shuzo, Kyoto). The PCR was performed with an UNO II thermocycler (Biometra, Germany). Primers used were as follows: SMS4-U, 5′-GATGGGCAGGAAGTGGTCAG-3′, and SMS4-D, 5′-CCCATGGAGTCAGGCTTCAG-3′ for *KCNQ1DN*; KIP7, 5′-GCCAAGTGCGCTGTGCTCGA-3′ and KIP2-23, 5′-CCTGCACCGTCTCGCGGTAG-3′ for *p57*^{KIP2}; ORCTL2-5, 5′-AGACGTCCCGAGGATCTTCC-3′ and ORCTL2-6, 5′-GAGACAGCCTTGATCAGCAT-3′ for *IMPT1*; IPL-5, 5′-GACCACAAGGAGATCGACTT-3′ and IPL-3, 5′-TCCTAGCCTCGGTCCGACTCGTCCAGCGTAT-3′ for *IPL*. PCR conditions were as follows: for *KCNQ1DN*, 45 cycles of 96°C for 45 s , 65°C for 30 s, 72°C for 25 s, followed by extension at 72°C for 2 min; for *p57*^{KIP2}, 40 cycles of 96°C for 30 s, 68°C for 30 s, 72°C for 25 s, followed by extension at 72°C for 2 min; for *IMPT1*, 40 cycles of 96°C for 30 s, 67°C for 30 s, 72°C for 45 s, followed by extension at 72°C for 2 min; for *IPL*, 40 cycles of 96°C for 30 s, 63°C for 30 s, 72°C for 45 s, followed by extension at 72°C for 2 min. As a control, β-*ACTIN* was amplified as follows: 25 cycles of 96°C for 45 s, 65°C for 30 s, 72°C for 25 s, followed by extension at 72°C for 2 min. Primers used were obtained from Takara Shuzo. The PCR products were electrophoresed on 2% agarose gel.

Identification of Transcribed Polymorphism in KCNQ1DN—To identify transcribed polymorphism in *KCNQ1DN*, both exons of *KCNQ1DN* were sequenced using three sets of primers in 15 fetal DNA samples. The primers used for the identification of the A703G polymorphism were as follows: SMS4-3, 5′-ATCCTGGTGAAGCCACACCC-3′, and SMS4-4, 5′-TGAGGCTGGCCGTTTAAAG-3′. The PCR was performed as follows: 35 cycles of 96°C for 45 s, 65°C for 30 s, 72°C for 30 s, followed by extension at 72°C for 2 min. The PCR products were pre-treated with exonuclease I and shrimp alkaline phosphatase according to the manufacturer's recommendations (Amersham Pharmacia, USA). The pre-treated PCR products were directly sequenced on both strands using BigDye system (Applied Biosystems, Germany) on an ABI 377 sequencer.

Analysis of Allele-Specific Expression of KCNQ1DN and IGF2—To examine the allele usage of *KCNQ1DN*, RT-PCR was performed using the RNA of two heterozygous fetal kidneys with the primer set SMS4-U and SMS4-D. The PCR conditions and sequencing were as described in the previous section. Similarly, to examine the parental origin of *KCNQ1DN* expression, the RT-PCR was carried out using the placental RNAs derived from the informative families. The genotyping of *IGF2* was performed by allele-specific PCR (*38*, *39*) depending on the known *Apa*I polymorphism (*40*). Allelic expression of *IGF2* was assessed using the same polymorphism, as described previously (*38*, *39*).

RESULTS

Identification of KCNQ1DN—The 244-kb genomic sequence derived from human chromosome 11p15.5 (GenBank accession no. AC001228, see Fig. 1A) was used to identify potential transcribed sequences and CpG islands, because this region was included within WT2. By BLASTN analysis, three expressed sequence tags (ESTs) (GenBank accession nos. AA828167, AI732937, and AI791256) between *p57*^{KIP2} and *KvLQT1* were identified; all were derived from the image clone 1422939. We have completely sequenced this clone and obtained a sequence of 1,109 bp. This cDNA had a poly(A) tail and a poly(A) signal (data not shown). Comparison of the cDNA sequence and the genomic sequence indicated that the 1,109-bp cDNA sequence contained two exons. We further screened the human testis cDNA library and obtained two additional cDNA clones. These clones were identical to the image clone in the exon-intron structure and poly(A) tail (Fig. 1B). To complete the 5′-end of this gene, we performed 5′ RACE using poly(A) RNA from fetal kidney, but no clones extending beyond the 5′-end of the image clone were obtained. The 5′-end of the 5′-RACE products was 30 bp shorter than that of the image clone (data not shown), suggesting that the 5′-end of the 5′ RACE products defined the 5′-terminus of major transcripts. Thus, the image clone could be derived from minor transcripts of the gene. This gene was designated as *KCNQ1DN* (*KCNQ1* downstream neighbor).

The accuracy of the cDNA sequences was confirmed not only by sequencing of the above three cDNA clones but also by sequencing of the RT-PCR product from the kidney. In addition, genomic PAC clone (accession number: AC-005950) also showed the identical sequence. From this sequence, we identified a short open reading frame (ORF) (encoding 68 amino acids) in *KCNQ1DN*, but no Kozak consensus sequence (*41*) around the ATG was identified, suggesting the *KCNQ1DN* gene has no efficient ORF.

We have also characterized the genomic DNA and found a CpG island with a direct repeat cluster upstream of

KCNQ1DN (Fig. 1B). This evidence suggests that the *KCNQ1DN* might be an imprinted gene (*42*).

Tissue-Specific Expression of KCNQ1DN—To assess the pattern of tissue specific expression of *KCNQ1DN*, we first performed Northern blot hybridization using human multiple tissue northern blots, but no hybridization signal was detected in various tissues (data not shown). However, RT-PCR analysis gave the product in adult brain, heart, kidney, testis, and placenta (Fig. 2). Also, the RT-PCR product was detected in all of nine human fetal kidney samples between 82 and 103 days of gestation (data not shown), suggesting constant expression in these tissues. These observations are important because the *KCNQ1DN* expression disappeared in Wilms' tumors, as described in a later section.

Maternal Expression of KCNQ1DN—Since *KCNQ1DN* was located within the imprinted domain of 11p15.5, we expected a mono-allelic expression of *KCNQ1DN*. The two exons of *KCNQ1DN* were amplified and sequenced from 15 individual fetal samples using three primer sets. One polymorphism (transition from A to G) was identified within exon 1 of *KCNQ1DN* at nucleotide 703 from the beginning of the cDNA image clone (Fig. 3A). Two of these samples were heterozygous for this polymorphism. To assess the imprinting status of *KCNQ1DN*, we carried out RT-PCR analysis of RNA derived from kidney of the two heterozygotes. By directly sequencing the PCR product which included the A703G polymorphism, both samples were shown to have mono-allelic expression of *KCNQ1DN* (Fig. 3B).

The same polymorphism was used to study parent allele–specific expression of *KCNQ1DN*. We analyzed 35 placental (villi, the genotype of which is the same as that of the embryo) DNAs and found 16 heterozygotes (data not shown). By typing the genomic DNA of available parents corresponding to the heterozygotes, three informative families were identified. In one family, genotypes were A/G (father), G/G (mother), and A/G (placenta). RT-PCR followed by direct sequencing showed that the placenta (villi) had mono-allelic expression (G allele), and subsequently the expressed allele was maternal (Fig. 3C). The other two

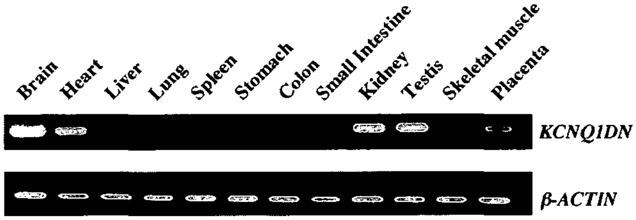

Fig. 2. **Tissue-specific expression of *KCNQ1DN*.** RT-PCR analysis demonstrates *KCNQ1DN* expression in different human adult tissues. RNA sources are indicated above the sample lanes. Primers used were SMS4-U and SMS4-D, which amplified a 330 bp fragment. β-*ACTIN* was the control and yielded a 275-bp fragment. Primers used and cycles of PCR are given in "MATERIALS AND METHODS."

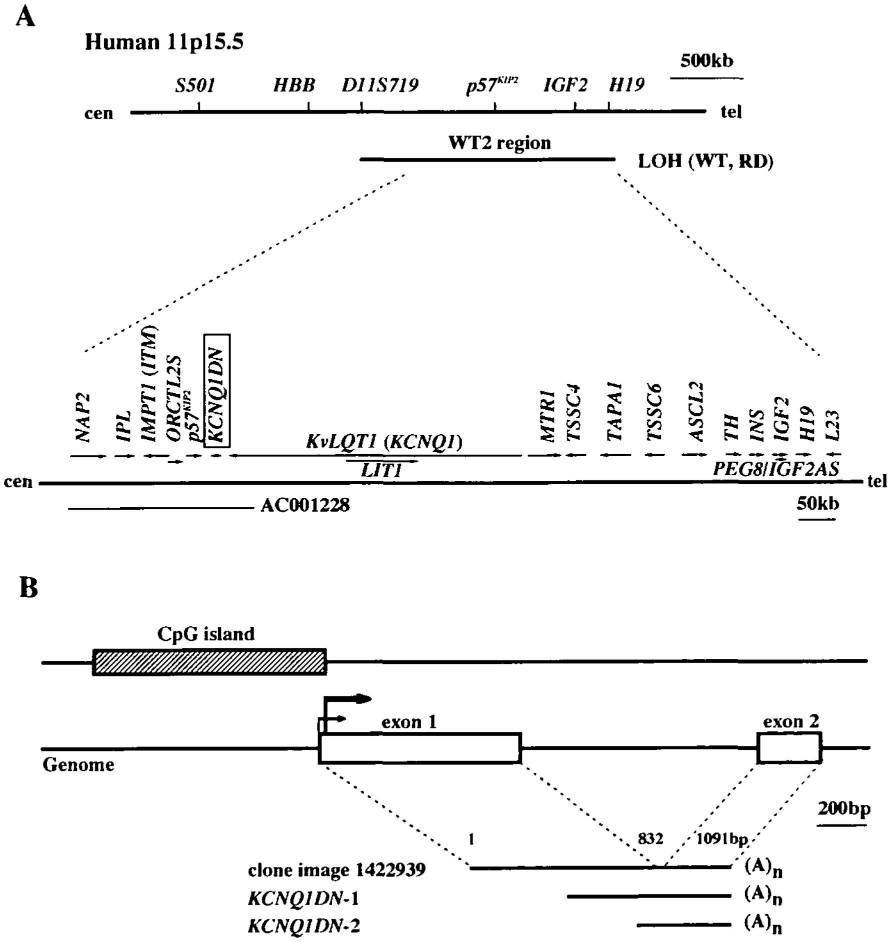

Fig. 1. **Mapping and structure of *KCNQ1DN* in human 11p15.5.** (A) Physical map of the 11p15.5 including the WT2 critical region (shown as LOH). The arrows indicate transcription orientation. LOH (WT, RD) indicates LOH in Wilms' tumor and in rhabdomyosarcoma. The gene identified here is enclosed with a square. The position of AC001228 used in this experiment is shown under the map. (B) Exon-intron structure of *KCNQ1DN* and the CpG island upstream of the gene. The IMAGE clone and two cDNA clones derived from human testis cDNA library are represented. Arrows represent the initiation site of the transcription. The thick and thin arrows imply the 5′-end of the major and minor transcripts, respectively. The 5′-end of minor transcript is tentative.

families also showed maternal expression (data not shown).

It has been reported that differentially methylated regions often overlap or are adjacent to direct repeat clusters in the vicinity of imprinted genes (*43*). No parent-of-origin–specific methylation was identified in the CpG island upstream of *KCNQ1DN* by a methylation–sensitive PCR assay (data not shown).

Expression of KCNQ1DN, $p57^{KIP2}$, IMPT1, and IPL in Wilms' Tumor—Since the maternally expressed gene, *KCNQ1DN*, is located within the WT2 critical region, it is a candidate for involvement in Wilms' tumorigenesis. We investigated the expression of *KCNQ1DN* in Wilms' tumors. Eighteen Wilms' tumors were investigated for the expression of *KCNQ1DN* by RT-PCR. Seven tumors showed no expression (Fig. 4 and Table I). Expression of other maternally expressed imprinting genes, including $p57^{KIP2}$,

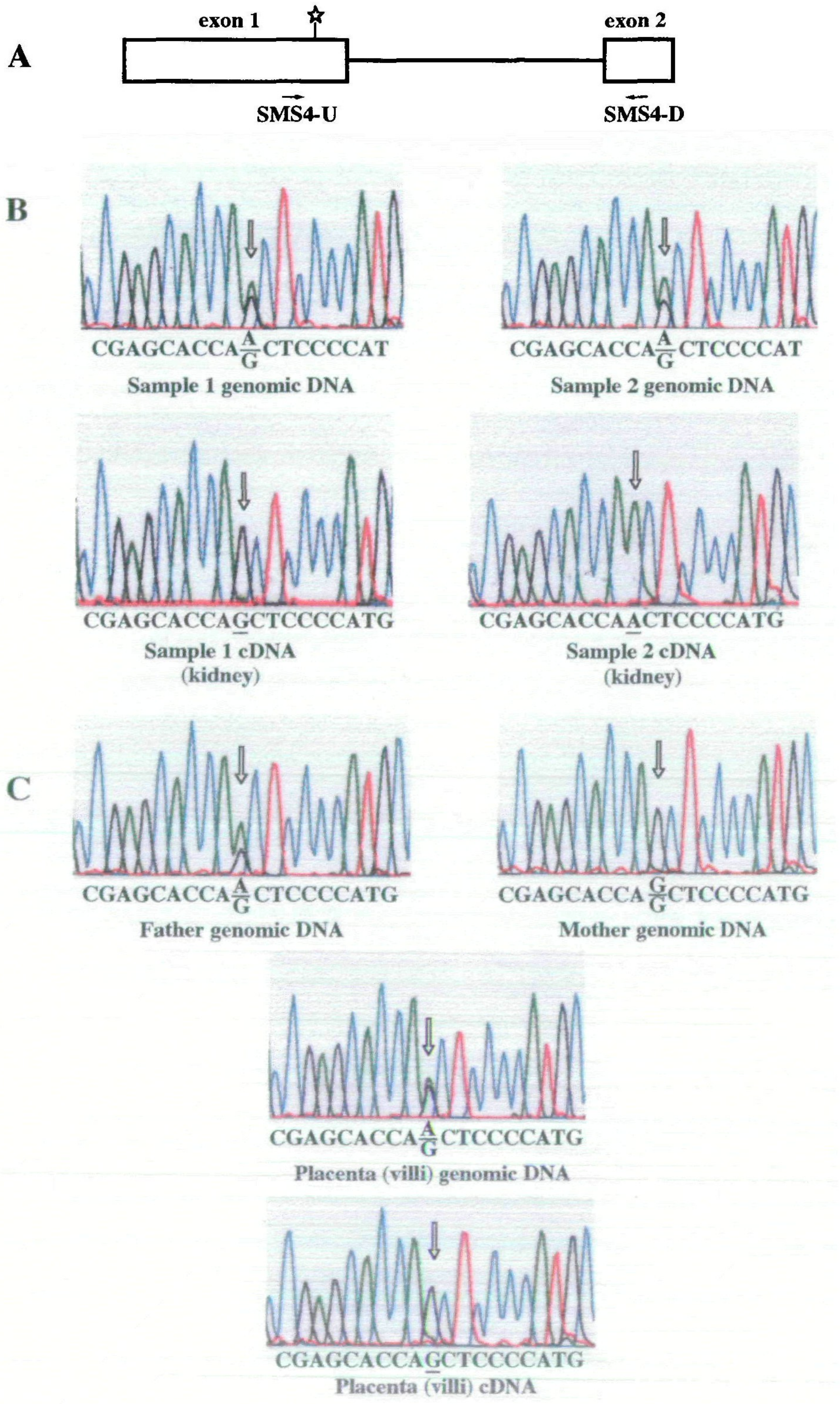

Fig. 3. **Mono-allelic and maternal expression of *KCNQ1DN*.** (A) Exon-intron structure and polymorphism within *KCNQ1DN*. The polymorphism at nucleotide 703 is indicated by an asterisk. Primers SMS4-U and SMS4-D were used for analysis of allele-specific expression. A product of 330 bp was generated. (B) Mono-allelic expression of *KCNQ1DN* in kidney of two fetuses. The polymorphic nucleotide is indicated by vertical arrows. (C) Maternal expression of *KCNQ1DN*. The genotype of the informative family and maternal expression of *KCNQ1DN* in placenta (villi) are shown. The polymorphism used for the analysis is the same as (A). The expressed nucleotide is indicated by a vertical arrow.

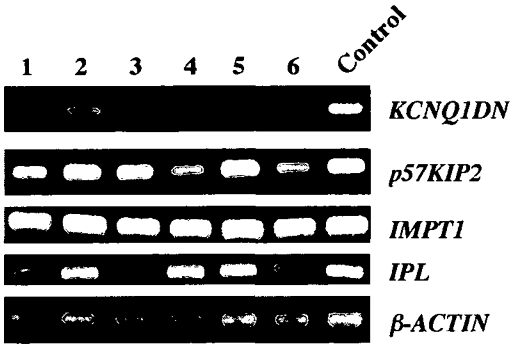

Fig. 4. **Expression of *KCNQ1DN*, *p57*^{KIP2}, *IMPT1*, and *IPL* in Wilms' tumors.** RT-PCR analysis demonstrated the expression of *KCNQ1DN*, *p57*^{KIP2}, *IMPT1*, and *IPL* in Wilms' tumor. The PCR product of each gene was amplified and detected by exon-connection PCR. Sample numbers are indicated above the sample lanes. These samples are also included in Table I with the same number. The control was normal fetal kidney. Primers used and PCR conditions are given in "MATERIALS AND METHODS."

TABLE I. **Expression of *KCNQ1DN* and imprinting status of *IGF2* in Wilms' tumor.**

Sample no.	Genotype of *KCNQ1DN*	Expression of *KCNQ1DN*	Imprinting status of *IGF2*	Genotype of *IGF2*	LOH of 11p
1	A/–	+		b/–	+
2	A/G	+	I	a/b	–
3	G/–	+		a/–	+
4	G/G	–		a/a	–
5	A/G	+	I	a/b	–
6	*	–		b/b	–
7	G/G	–		b/b	–
8	G/G	–		a/a	–
9	*	+		a/a	–
10	A/G	+	LOI	a/b	–
11	A/G	+	LOI	a/b	–
12	G/G	+		b/b	–
13	*	–		a/a	–
14	G/G	+		a/a	–
15	A/G	+	LOI	a/b	–
16	A/G	–	LOI	a/b	–
17	A/G	–	LOI	a/b	–
18	A/G	+	I	a/b	–

I: Normal imprinting. LOI: Loss of imprinting. LOH: Loss of heterozygosity (by cytogenetic analysis). a: Allele not including *Apa*I site (GGACCC). b: Allele including *Apa*I site (GGACCC). *: Not amplified.

IMPT1, and *IPL* in these tumors was also investigated. All were shown to be normally expressed in these samples (Fig. 4 and data not shown).

Imprinting Status of IGF2 in Wilms' Tumor—The silencing of the maternal allele of *H19* was accompanied by biallelic activation of the linked and reciprocally imprinted *IGF2* gene in some Wilms' tumors (*29*). We examined whether the silencing of the maternal allele of *KCNQ1DN* is also accompanied by biallelic activation of *IGF2*. We genotyped Wilms' tumors using the A703G polymorphism for *KCNQ1DN* and the *Apa*I polymorphism for *IGF2* (*40*). Eight tumors were heterozygous (informative) for both *KCNQ1DN* and *IGF2* (Table I). We next determined the imprinting status of *IGF2* (*38*) in informative samples. Among six samples with expressed *KCNQ1DN*, three showed biallelic expression of *IGF2* and the others had mono-allelic expression. In contrast, two other tumors with no expression of *KCNQ1DN* all exhibited biallelic expression of *IGF2* (Table I).

DISCUSSION

WT2 is defined by maternal-specific loss of heterozygosity (LOH) of chromosome 11p15.5 in Wilms' and other embryonal tumors, as well as in a variety of adult tumors, suggesting that imprinted genes in this region are involved in these cancers (*11–16*). In addition, molecular data including experiments involving the transfer of a subchromosomal fragment into the tumor cell line, showed that 11p15.5 harbors at least one tumor suppressor gene (*37*). Several imprinted genes in this region have been well studied for possible involvement in Wilms' tumorigenesis, especially *H19*, *IGF2*, *p57*^{KIP2}, and *IMPT1*, but these genes could not account for all Wilms' and other tumors. Therefore, other genes in the WT2 region must be responsible for these tumors. By BLAST analysis and CpG island search using the 244 254 bp genomic sequence of human chromosome 11p15.5, we identified a novel imprinted gene, *KCNQ1DN*, which is located within the WT2 critical region and showed maternal expression. The expression of KCNQ1DN was reduced in 7 of 18 (39%) Wilms' tumors. In addition, those Wilms' tumors with loss of expression of *KCNQ1DN* showed normal expression of *p57*^{KIP2}, *IMPT1*, and *IPL*, and had no LOH of 11p (Table I), suggesting that the maternally expressed *KCNQ1DN* was involved in Wilms' tumors. These results also suggest that the loss of the *KCNQ1DN* function might play some role in Wilms' tumors in which the WT2 region is involved. Further functional analysis will be required.

Human chromosome 11p15.5 has been hypothesized to have two imprinted subdomains: the telomeric domain including *IGF2* and *H19*, alterations of which may be more specific for malignancy; and the centromeric domain including *p57*^{KIP2}, *KvLQT1*, and *LIT1*, alterations of which may be more specific for BWS (*44*). Our finding of a reduced expression of *KCNQ1DN* in Wilms' tumors suggests that *KCNQ1DN* within the centromeric domain is also involved in tumorigenesis. We also attempted to clarify the relationship between the expression of *KCNQ1DN* and the imprinting status of *IGF2*. Although both informative samples with loss of expression of *KCNQ1DN* exhibited LOI of *IGF2*, we could not confirm the relationship between them because of the small number of informative samples. Further examination of a large number of Wilms' tumors will be required.

Recently, a paternally expressed imprinted gene, *PEG8/IGF2AS* in the telomeric domain of 11p15.5, has been reported to be overexpressed in Wilms' tumors (*22*). In contrast to the overexpression of the two paternally expressed imprinted genes, *IGF2* and *PEG8/IGF2AS*, the reduced expression of the maternally expressed imprinted gene, *KCNQ1DN*, in Wilms' tumors was observed. These observations are highly compatible, assuming that overexpression of paternally expressed imprinted genes and loss of maternally expressed repressor genes could be responsible for Wilms' tumors as well as BWS.

LIT1, an antisense RNA of *KvLQT1*, has a CpG island with a maternally methylated region near the 5′ end of this transcript (located in intron 10 of *KvLQT1*). Mitsuya *et al.*

(*20*) and Lee *et al.* (*21*) proposed that this CpG-rich sequence might work as an insulator like that of the *H19* and *IGF2* region proposed by Thorvaldsen *et al.* (*45*). On the maternal chromosome, *KvLQT1* would be activated instead of *LIT1*, because the maternal CpG sequence would be methylated. On the paternal chromosome, however, *LIT1* would be activated instead of *KvLQT1*, because there is no methylation at the CpG sequence. In this case, the authors thought that on the maternal chromosome, the enhancer of the *KvLQT1* would affect not only itself but also other downstream target-genes like a $p57^{KIP2}$ across the inactive insulator due to the methylated CpG sequence, but that on the paternal chromosome, on the contrary, the enhancer of the *KvLQT1* would affect the *LIT1* but not the downstream target-genes because of the active insulator due to the absence of methylation of the CpG sequence. They claimed that this was why the $p57^{KIP2}$ from the paternal chromosome was not expressed. *KCNQ1DN* might have lost its activity for the same reason on the paternal chromosome, because this gene was maternally, but not paternally, expressed and, in addition, it was located closer to the *LIT1* than the $p57^{KIP2}$.

They also observed that the maternal methylation of the CpG sequence was eliminated in BWS patients, allowing *LIT1* to have biallelic expression. These facts suggested that the insulators of both alleles were active, and that the maternal $p57^{KIP2}$ allele would not be expressed in BWS patients, although this has not been confirmed yet. If this is the case, it is possible that the *KCNQ1DN* might not be expressed like the $p57^{KIP2}$, as for the BWS patient carrying biallelic expression of the *LIT1*. They also analyzed Wilms' tumors and observed normal expression of the *LIT1* in them, suggesting that *LIT1* is not involved in Wilms' tumorigenesis. However, according to the insulator model in the *KvLQT1/LIT1* region, *KCNQ1DN*, as well as $p57^{KIP2}$, might be regulated in the same way as explained above. If this hypothesis is correct, it will be intriguing to examine whether the maternal methylation of the CpG sequence of *LIT1* disappears in the tumor carrying no expressed *KCNQ1DN*. This possibility is currently under investigation.

We wish to thank Dr. A. Fantel, Ms. M. Eisenhauer, and Mr. J. Rajan, Birth Defects Research Laboratory, University of Washington, for providing the fetal tissues, and Dr. L. Filippi for pertinent advice. We also thank all the other members of the Department of Biochemistry at Saga Medical School for their critical comments.

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