Genomic imprinting of human p57\textsuperscript{KIP2} and its reduced expression in Wilms’ tumors

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\textbf{p57\textsuperscript{KIP2} is a potent tight-binding inhibitor of several G\textsubscript{1} cyclin complexes, and is a negative regulator of cell proliferation.} The gene encoding human p57\textsuperscript{KIP2} is located on chromosome 11p15.5, a region implicated in both sporadic cancers and Beckwith–Wiedemann syndrome (BWS), a cancer syndrome, making it a tumor suppressor candidate. Several types of childhood tumors including Wilms’ tumor, adrenocortical carcinoma and rhabdomyosarcoma display a specific loss of maternal 11p15 alleles, suggesting that genomic imprinting plays an important part. Genetic analysis of the familial BWS has indicated maternal carriers and suggested a role in genomic imprinting. Previously, we demonstrated that p57\textsuperscript{KIP2} is imprinted in the mouse. Here we describe the genomic imprinting of human p57\textsuperscript{KIP2} and the reduction of its expression in Wilms’ tumors. High resolution mapping locates p57\textsuperscript{KIP2} in the region responsible for both tumor suppressivity and BWS.

\textbf{INTRODUCTION}

Genomic imprinting is the parental-allele-specific expression of genes. In mammals, genomic imprinting ensures functional inequality of paternal and maternal genomes in the fertilized egg and causes developmental failure of embryos produced by parthenogenesis or by gynogenesis or androgenesis (1–6). Parental effects on particular chromosomal regions involving embryo survival and gross phenotypic abnormalities have been unequivocally documented by producing paternal or maternal disomies by means of Robertsonian and reciprocal translocations in the mouse (7). Such studies have shown that several autosomal chromosomes are concerned in imprinting. The basis for the developmental failure and the phenotypic abnormalities have been attributed to the imprinting of specific genes (8–10).

Progression through the cell cycle is catalyzed by cyclin-dependent kinases (CDKs) and is negatively controlled by CDK inhibitors (CDKIs). p57\textsuperscript{KIP2} is related to p21\textsuperscript{CIP1} and p27\textsuperscript{KIP2}, and is a potent tight-binding inhibitor of several G\textsubscript{1} cyclin/CDK complexes (11,12). Overexpression of p57\textsuperscript{KIP2} arrests cells in G\textsubscript{1}. The gene encoding human p57\textsuperscript{KIP2} is located on chromosome 11p15.5 (12), a region implicated in both Beckwith–Wiedemann syndrome (BWS) and sporadic cancers.

BWS is characterized by numerous growth abnormalities, including macroglossia, gigantism, visceromegaly, exomphalos and an increased risk of childhood tumors, including Wilms’ tumor, adrenocortical carcinoma, rhabdomyosarcoma and hepatocellular carcinoma (13). Although most cases of BWS are karyotypically normal and sporadic, including Wilms’ tumor, adrenocortical carcinoma, rhabdomyosarcoma and hepatocellular carcinoma (13). Although most cases of BWS are karyotypically normal and sporadic, there are patients with chromosome 11 duplications (14,15) or translocations (16) and a few families with autosomal dominant transmission (17). Evidence that the gene for BWS is likely imprinted comes from the increased paternal transmission pattern seen in the autosomal dominant type pedigrees (18,19) and especially from the findings of paternal uniparental disomy (UPD) reported for a subgroup of patients (20). The region most commonly involved in uniparental disomy includes the gene for p57\textsuperscript{KIP2} on 11p15.5 (20). The gene for BWS has also been localized to the 11p15.5 region by linkage analysis of autosomal dominant pedigrees (21,22).

Loss of heterozygosity at 11p15.5 has been observed in a number of human cancers including breast cancer, bladder, lung, ovarian, kidney and testicular carcinoma (23). Several types of childhood tumors, including Wilms’ tumor, adrenocortical carcinoma, rhabdomyosarcoma and hepatocellular carcinoma, display a specific loss of maternal 11p15 alleles, suggesting that genomic

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imprinting plays an important part (23–26). Through experiments using subchromosomal transferable fragments from 11p15, a tumor suppressor gene has been mapped to the vicinity of D11S724 and D11S719, excluding H19 (27). These facts suggest that p57KIP2 is an imprinted tumor suppressor in this locus.

We proved the genomic imprinting of mouse p57KIP2 previously (28). Here we describe the genomic imprinting of human p57KIP2 and its expression in Wilms’ tumors. High resolution mapping locates p57KIP2 in the region responsible for both tumor suppressivity and BWS.

RESULTS

Genomic structure of human p57KIP2

The PCR of human genomic DNA with primers (primers A and B) designed to amplify the 3’ non-coding sequence of cDNA gave a 279 bp fragment. This fragment was used to screen a human genomic library. Six clones were obtained and one of them was used for analysis. Sequence analysis of this clone and comparison with previously reported cDNA sequence (12) revealed two small introns and their exon/intron junctions (Fig. 1).

Discrimination between parental alleles of p57KIP2

To discriminate between the parental alleles of p57KIP2, we searched for length polymorphisms in the repeated region by PCR. We screened genomic DNA derived from peripheral blood using the primers C and D, and found a length polymorphism. We could detect a 103 bp fragment and 91 bp fragment in lane 3 (Fig. 2). This sample is a heterozygote. Sequence analysis of both the 103 bp and 91 bp fragments revealed a 12 bp in-frame deletion in the p57KIP2 open reading frame (Fig. 1).

Monoallelic expression of p57KIP2

To identify samples heterozygous for the length polymorphism, we first screened genomic DNA derived from the kidneys. Of six samples, one was heterozygous and five were homozygous for the 103 bp fragment. We next examined the presence of each allele in RNA transcripts from liver, kidney and skeletal muscle thus identified as heterozygous. The majority of RNA transcripts were from one allele, but slight expression of another allele also could be detected in these tissues (Fig. 3). We similarly examined RNA transcripts from the brain and liver of a fetus and three placenta of different stages (11–26 weeks of gestation). In fetal liver and placenta, the majority of RNA were from one allele. In the fetal brain, although the majority of RNA were from one allele, a large amount of another allele could be detected (Fig. 3).

Parental origin of the expressed allele

To assign the parental origin to the expressed allele, we examined the genomic DNA of peripheral blood of available parents of corresponding fetuses. Of 61 fetuses, we identified two informative families. Figure 4a shows that the expressed allele has been derived from the mother. To further confirm the parental origin of the expressed allele, we utilized another sequence polymorphism which could be detected by direct sequencing of PCR product (Fig. 4b). By using this, we could also demonstrate that the maternal allele was expressed (Fig. 4c).

Reduced expression of p57KIP2 in Wilms’ tumors

If p57KIP2 is involved in Wilms’ tumors, the expression level of the gene will be expected to be reduced. Therefore, Wilms’ tumors were examined for the expression of p57KIP2 by using a quantitative RT-PCR assay (29). Of seven tumors examined, three showed reduced expression of p57KIP2 (approximately ten-fold reduced from the level in normal kidney) in contrast to the equal level of expression of control S14 ribosomal protein gene (Fig. 5).
Figure 4. Parental origin of expressed allele. (a) Placenta RNA from heterozygous children was subjected to RT-PCR assay with (+) and without (−) reverse transcriptase. The primers used were C and D in Figure 1. Genomic DNA from the children, father and mothers was analyzed with PCR. 103 bp and 91 bp indicate fragment length of each allele. (b) The polymorphism was detected by direct sequencing of PCR product. The downward arrows indicate the site of the polymorphism (at position 815 of the cDNA). (c) Placenta RNA from heterozygous child was subjected to RT-PCR followed by direct sequencing. Genomic DNA from the child, father and mother was analyzed by direct sequencing of PCR product. The primers used from RT-PCR and PCR were G and H. Direct sequencing was performed with primer G.

Figure 5. Expression of p57KIP2 in Wilms’ tumors. RNA from Wilms tumors (lanes 1–14) and normal kidney (lanes 15–18, lane 15 and 16 were normal kidney of lane 9 and 10) was subjected to quantitative RT-PCR assay (29) with (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17) and without (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18) reverse transcriptase. Primers used for RT-PCR were: E and F, for p57KIP2: 5′-GGCA-GACCGAGATGAATCCCTCA-3′ and 5′-CAGGTCCAGGGTCTTGGTCC-3′, for S14 ribosomal protein gene.

High-resolution cytogenetic mapping of p57KIP2

Experiments using subchromosomal transferable fragments from 11p15 have provided evidence that a tumor suppressor gene of rhabdomyosarcoma resides in the vicinity of D11S724 and D11S719 (27). The breakpoints in the BWS patients were mapped to the interval between cC15–19 and q1 (30). These rearrangements seem to indirectly affect neighboring genes by some unknown type of position effect, perhaps genomic imprinting, because these breakpoints span a considerably larger genomic distance. Therefore, the gene for BWS should be located in the vicinity of cC15–19 and q1. If the tumor suppressor gene and BWS gene are identical, these findings suggest that the most likely position of the gene is located in the vicinity of D11S724 and q1 (Fig. 6a). High-resolution cytogenetic mapping (Fig. 6b,c) indicated that p57KIP2 is located very close to D11S679 which is between D11S724 and q1. The physical distance between D11S679 and p57KIP2 is less than 40 kb.

DISCUSSION

Our data demonstrate that, in the adult and fetal tissues studied, p57KIP2 mRNA is expressed mostly from one allele, although slight expression of another allele also could be detected in most tissues and a large amount of another allele could be detected in the fetal brain. Where the parental origin of the expressed allele could be ascertained, it was maternal. Thus, p57KIP2 is parentally imprinted in both humans and mice. The amount of expression of repressed allele is different between humans and mice (28). Slight expression of repressed allele could be detected in humans although one could not be detected in mice. This difference could come from a difference in the amount of allelic methylation. The allelic methylation in the mouse was easily found in the gene body whereas that in the humans still could not be found (29 and unpublished results). The maternal expression of p57KIP2 can explain the maternal transmission of BWS and loss of the maternal alleles in childhood tumors.

We showed reduced expression of p57KIP2 in Wilms’ tumors. We also showed that the p57KIP2 gene was transcribed predomi-
nantly from the maternal allele in normal tissues. These findings suggest that loss of the maternal alleles or aberrant imprinting of the maternal alleles reduced the expression of p57\textit{KIP2} in Wilms’ tumors. The four Wilms’ tumors whose expression of p57\textit{KIP2} was not reduced could be due to other tumor suppressors. Loss of heterozygosity at chromosome 16 was also observed in Wilms’ tumors (30). The tumor suppressor gene region defined by subchromosomal transferable fragments contains one cluster of BWS breakpoints but excludes the breakpoints more than 4 Mb centromeric to this region, as well as H19. Most likely, a group of cancer-related genes fall within a several megabase region, similar to 1p, 3p and 9p (31).

A rhabdomyosarcoma tumor suppressor gene was mapped to the vicinity of D11S724 and D11S719 using subchromosomal transferable fragments from 11p15 (27). This region excludes an imprinted gene H19 (28). The breakpoints of BWS patients mapped to the vicinity of cC15–19 and q1 (32). If the tumor suppressor gene and BWS gene are identical, these findings suggest that the most likely position of the gene is located in the vicinity of D11S724 and q1 (Fig. 6a).

High-resolution cytogenetic mapping indicated p57\textit{KIP2} is located very close to D11S679 which is between D11S724 and q1 (Fig. 6). This also suggests that p57\textit{KIP2} is the BWS gene and tumor suppressor in this region.
MATERIALS AND METHODS

Sequencing

Genomic clone was subcloned into pBluescript and the sequence around the mRNA coding region was determined by using a Taq dideoxynucleotide kit (ABI).

RT-PCR

Total RNA, prepared by the acid-phenol method (33) was applied to RT-PCR as described (34). The primer sequences used were: A, 5′-CCACATTAGGCCCCAGG-3′; B, 5′-TGATCTGAGGTTCCAGCAGA-3′; C, 5′-CCACCCCGCCCCCATCC-3′; D, 5′-GGGGGCGGACCCGACC-3′; G, 5′-CGGATTTCCGGACGGCTGCT-3′; H, 5′-CCAAAGCTTTGCTCCGGCCTTCTTGAAG-3′.

Quantitative RT-PCR assay

Quantitative RT-PCR assay was performed as described (29) except that detection was done by Southern blotting. Primers used were: E, 5′-GGGGCGCATTAGGCCCCAGG-3′; F, 5′-CCGTTGTTGCTCATGAAAC-3′; S, 5′-ribosomal protein sense, 5′-GCCGACGGATTTCCGACGAGATGAC-3′; S14 ribosomal protein anti-sense, 5′-CACCCAGCTTTGCTCCGGCCTTCTTGAAG-3′.

Localization of DNA markers by multicolor FISH

For high-resolution ordering by multicolor FISH, elongated prophase chromosomes were obtained from cultured lymphocytes by the thymidine synchronization, BrDU release technique, in the presence of the topoisomerase II inhibitor ICRF154 as previously described (35). Before hybridization in situ, chromosomes were stained in Hoechst 33258 and irradiated with UV according to Takahashi et al. (36) with minor modifications. Stretched DNA slides were prepared as described (37).

Multicolor FISH was performed as previously reported (38). In brief, the first and second probes were labeled with bio-16-dUTP (Boehringer), and the second and third probes with digoxigenin-11-dUTP (Boehringer) by nick translation. Labeled probes were precipitated with sonicated salmon sperm DNA and E. coli tRNA, and were dissolved in 20 µl of formamide. Bio- and dig-labeled second probe solutions were mixed in a ratio of 7:3, and the mixture was used as a bio/dig dual probe instead of a double-labeled probe. In the hybridization with three different probes, the solutions of first, second and third probes were mixed in a ratio of 1:5:2:5:6.0 (v/v). To eliminate background noise due to repetitive sequences of rRNA, and L1, 0.6 µl of sonicated human tRNA, and were dissolved in 20 µl of formamide. Bio- and dig-labeled second probe solutions were mixed in a ratio of 7:3, and the mixture was used as a bio/dig dual probe instead of a double-labeled probe. In the hybridization with three different probes, the solutions of first, second and third probes were mixed in a ratio of 1:5:2:5:6.0 (v/v). To eliminate background noise due to repetitive sequences of Alu and L1, 0.6 µl of sonicated human placental DNA (10 mg/ml) was added to 9.4 µl of the triple-probe solution. The final mixture was denatured at 70°C for 5 min and mixed with an equal volume of 4× SSC with 20% dextran sulfate. The hybridization mixture was placed on denatured slides, covered with Paraflm, and incubated in a humid box at 37°C for 16–18 h. After being washed in 50% formamide/2× SSC, 2× SSC, and 1× SSC (37°C, 15 min, each), the slides were treated with 4% Block Ace (Dainippon Pharmaceutical Co. Ltd) at 42°C for 15 min to block fluorochrome background noise. They were then incubated in 4× SSC with 1% Block Ace containing activin-FITC (5 µg/ml) (Boehringer) and anti-digoxigenin rhodamine (1 µg/ml) (Boehringer) at 37°C for 40 min, and then washed for 10 min in each of 4× SSC, 4× SSC/0.05% Triton X-100, and 4× SSC. Then, the slides were counterstained with DAPI (1 µg/ml) and mounted in an anti-fade solution containing 1% DABCO (1,4-diazabicyclo[2.2.2]octane) (Sigma).

Microscopy was performed with a Nikon FXA epifluorescence microscope. Both metaphase chromosomes on stretched DNA fiber stained with DAPI were screened through a Nikon UV-2A filter (exciter, 400–440 nm; barrier, 470 nm). To estimate the signal conditions for each probe, FITC and rhodamine signal were observed with filters of B-2E (exciter, 450–490 nm; barrier, 520–560 nm) and G-2A (exciter 510–560 nm; barrier 590) respectively. Signals of the three probes were simultaneously visualized through a double band-pass filter (excitation centers, 490 nm and 560 nm; emission centers, 530 nm and 650 nm) (Omega Optical). The microphotographs were taken using Fujichrome 400D film exposed at 1600 ASA. The photoslides were projected onto a screen, and the distances between the centers of the fluorescent signals were measured (mm).

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