Lack of Imprinting of Three Human Cyclin-Dependent Kinase Inhibitor Genes

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ABSTRACT

Genomic imprinting is an epigenetic modification in the germ line leading to parental allele-specific gene expression in somatic cells. We have previously found that imprinted genes can be abnormally expressed or silenced in tumors and that the cyclin-dependent kinase inhibitor (CKI) CDKN1C (p57KIP2) is normally imprinted, with preferential expression of the maternal allele. Here we analyze the imprinting status of three additional CKIs, the abnormal expression and/or chromosomal localization of which has been implicated in human malignancy: CDKN1A, CDKN1B, and CDKN2C. Allele-specific expression was examined by reverse transcription-PCR, using primers that span transcribed polymorphisms as well as exon/intron boundaries, to distinguish cDNA products from genomic DNA. Biallelic expression was observed for all three genes in both fetal and adult tissues. Thus, genomic imprinting is not a generalized feature of CKIs.

INTRODUCTION

Genomic imprinting is defined as an epigenetic modification of a specific chromosome in the gamete or zygote leading to differential expression of maternal and paternal alleles in the somatic cells of the offspring. Loss of imprinting in cancer can lead to abnormal expression of growth-stimulatory genes, as well as epigenetic silencing of growth-inhibitory genes (for a review, see Ref. 1). A small number of human genomic regions have been identified that harbor multiple imprinted genes. One of these is 11p15.5, which includes IGF2, expressed from the paternal allele, and H19, expressed from the maternal allele. We and our collaborators (2) recently localized the CKI gene CDKN1C (p57KIP2) to the same 700-kb genomic region and found that this gene is also imprinted, with preferential expression of the maternal allele. We also found that CDKN1C, like IGF2 and H19, can undergo abnormal imprinting and expression in cancer (3).

CDKN1C is the most recently identified member of a class of growth-regulatory factors called CKIs. CKIs function by binding to cyclin-dependent kinases and inactivating their proliferative activity. CDKN1C shares conserved domains with CDKN1A (p21WAF1/CIP1), a target of p53-mediated growth arrest (4). CDKN1A also undergoes somatic mutation in a Burkitt’s lymphoma (5). CDKN1C is also structurally related to CDKN1B (p27KIP1), which has been found only rarely to be mutated in human tumors (6) but is localized to chromosomal band 12p13, which shows LOH in hematopoietic malignancies (7) and non-small cell lung cancer (8). Members of the INK4 family of CKIs share homologous domains with each other but differ from CDKN1A, CDKN1B, and CDKN1C. Four of the INK4 genes, CDKN2B (p15), CDKN2A (p16), CDKN2C (p18), and CDKN2D (p19) exhibit ankyrin repeat motifs (9). CDKN2C is localized to chromosomal band 1p32, and 1p shows preferential loss of the maternal allele in neuroblastoma, suggesting that a tumor suppressor gene on this band is imprinted (10). In this study, we examined the possibility that imprinting might be a common regulatory mechanism of CKIs, focusing on the two genes most closely related to CDKN1C, namely CDKN1A and CDKN1B, as well as one of the INK4 family members, CDKN2C. Table 1 summarizes the CKIs studied here.

MATERIALS AND METHODS

Identification of Individuals Heterozygous for Restriction Site Polymorphisms. Samples from 22 fetuses were obtained from the University of Washington Fetal Tissue Bank, and 16 adult tissue samples were obtained from snap-frozen surgical specimens. Genomic DNA was isolated as previously described (2). PCR was performed in Hot Start Micro 50 reaction tubes (Molecular Bio-Products) in a volume of 50 μl. Reactions contained 20 μM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.25 mM each dNTP, 2.5 units of Taq polymerase (Life Technologies, Inc.), 100 ng of genomic DNA, and 0.5 μM each of the appropriate primers. Amplifications were performed in a Stratagene Robocycler and consisted of an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C (CDKN1A), 58°C (CDKN1B), or 62°C (CDKN1B) for 45 s, and extension at 72°C for 90 s. After cycling, the reactions were brought to 72°C for 10 min, then cooled to 6°C. The primers used were as follows: CDKN1A-7, 5’-CCCAGGGAATGGGGTTCCTG-3’ (forward); CDKN1A-8, 5’-GGCGGCCAGGGTATGTAC-3’ (reverse); CDKN1B-3, 5’-GGCGGCCAGGGTATGTAC-3’ (reverse); CDKN1B-2, 5’-TGCCAGGAGACTGCTACTTACAGAGG-3’ (reverse); and CDKN2C-3, 5’-TTATGGAGATCTGGGAGCCC-3’ (reverse). Amplified products of the CDKN1A, CDKN1B, and CDKN2C loci were digested with 20 units of PstI, BglII, and BsrEII, respectively (New England Biolabs). DNA fragments were electrophoresed in Tris-Acetate-EDTA buffer through 2% electrophoresis-grade agarose (Life Technologies, Inc.) and SeaKem agarose (FMC Bioproducts).

Assay for Allelic Expression of CKIs by RT-PCR. RNA from heterozygous individuals was obtained as described previously (2) or by isolation with RNAzol B (Tel-Test Inc.). According to the manufacturer’s instructions, cDNAs were synthesized in a reaction volume of 25 μl by incubating RNA, 0.5 units of RNase inhibitor (Promega), and 20 units of DNase I (Pharmacia) in 1× first-strand buffer (Life Technologies, Inc.) for 90 min at 37°C. DNase I was subsequently thermally inactivated by heating to 94°C for 10 min. After oligo(dT)15 (Boehringer Mannheim; CDKN1A and CDKN2C reactions) or CDKN1B-S3 (CDKN1B reaction) was added to a final concentration of 20 ng/μl, the reaction was heated to 72°C for 10 min and then cooled on ice. DTT was added to a final concentration of 8 mM, dNTPs to a final concentration of 0.4 mM, and RNase inhibitor to a final concentration of 0.04 unit/μl. The mixtures were then incubated at 42°C for 2 min, 1 μl (200 units) of Superscript II Reverse Transcriptase (Life Technologies, Inc.) was added, and the reactions were allowed to proceed for 60 min at 42°C. The reaction was terminated by the addition of 1 unit of RNase H (Life Technologies, Inc.) followed by incubation at 37°C for 20 min. cDNAs were purified by phenol-chloroform extraction and ethanol precipitation and resuspended in 1× PCR buffer (Life Technologies, Inc.). Two-μl aliquots of the synthesized cDNAs were used for amplification by PCR as described above, with the addition of seven cycles (42 cycles total) to the procedure, except for CDKN1A, for which 30 or 35 cycles were used, to avoid heterodimer formation. Oligonucleotide primers used for PCR were designed to distinguish cDNA-derived PCR products from that of contaminating genomic DNA and were as follows: CDKN1A-9, 5’-CACCGGACAGCAGAGGACAC-3’ (forward); CDKN1A-8 (reverse); CDKN1B-3, 5’-ACATTTTCTCTGTGTTGG-3’ (reverse); CDKN2C-1, 5’-TGTAAAGCTCAATAGGACAAATGG-3’ (reverse); CDKN2C-2, 5’-GCTGGAGGGAGTTGCTCTAGG-3’ (reverse); CDKN2C-3, 5’-TTATGGAGATCTGGGAGCCC-3’ (reverse); and CDKN2C-4, 5’-TGTACGCTGTAGAGACAGG-3’ (reverse).
RESULTS AND DISCUSSION

Analysis of CDKN1A. To assay allele-specific expression of CDKN1A, we designed primers that spanned a known PstI polymorphism in exon 3 (11). To identify patients heterozygous for the polymorphism, primers were designed within intron 2 and exon 3 (see “see Materials and Methods”). DNA was extracted from 22 samples from separate fetal specimens, PCR was performed, and the products were digested with PstI. From this analysis, two fetuses were identified as being heterozygous for the PstI site. The overall frequency of the noncutting allele was observed to be 6%, very close to the 9% frequency observed by Law and Deka (11). RNA was prepared from the lungs, intestines, liver, and kidneys of each of these fetuses, reversed transcribed, and PCR amplified using primers spanning intron 2 and the PstI polymorphism (Fig. 1). PCR products were digested with PstI and analyzed by gel electrophoresis. In all tissues examined, biallelic expression was observed (Fig. 2). To confirm the apparent similarity of expression of the two alleles, DNA was amplified in an analogous manner, transferred by Southern blotting, and hybridized with an internal probe for CDKN1A. The filter was then subjected to PhosphorImager analysis, which revealed identical amplification of both DNA and RNA. Thus, CDKN1A showed no evidence of imprinting in two independent fetuses.

To rule out the possibility that genomic imprinting might be manifest as allele-specific expression postnatally, 16 additional adult colons were analyzed for heterozygosity of the polymorphism, of which 3 heterozygotes were identified. RNA was extracted from these three samples and analyzed as described above. As observed in the fetal tissue samples, biallelic expression was seen in each case (Fig. 2). Thus, there was no evidence for genomic imprinting of CDKN1A.

Analysis of CDKN1B. CDKN1B, like CDKN1A, shares homology with the CKI domain of CDKN1C and was therefore analyzed for evidence of genomic imprinting. To assay allele-specific expression, primers in exon 1 and intron 1 were designed that spanned a known BgII polymorphic site (Ref. 6; Fig. 1). Analysis of DNA from 12 fetal specimens and 16 adult specimens identified 1 fetal specimen and 5 adult specimens that were informative for this polymorphic marker. RNA was extracted from liver, heart, placenta, and colon, and subjected to RT-PCR and BgII digestion. The results of these experiments performed in duplicate showed biallelic expression in all tissues examined. Again, Southern hybridization performed with a probe internal to CDKN1B followed by PhosphorImager analysis revealed equal expression of both alleles of the gene (Fig. 2). Thus, no evidence for genomic imprinting was found.

Analysis of CDKN2C. A similar approach was used for CDKN2C as for CDKN1A and CDKN1B. In this case, primers were used that spanned intron 1 and a polymorphic BstEII site (12). Three of 22 fetuses were heterozygous for this polymorphism, corresponding to a 7% allele frequency. All tissues examined (including the liver, lung, and placenta) from this individual showed biallelic expression, and quantitation again showed equal representation of both alleles (Fig. 2).

We had shown previously that the human CDKN1C gene is normally imprinted with preferential expression of the maternal allele (2), suggesting that genomic imprinting might be a regulatory feature of functionally related genes. The major result of this study is that at least three other CKIs, two of which (CDKN1A and CDKN1B) show strong homology to CDKN1C, show no evidence of imprinting. All three genes studied map to chromosomal regions that show LOH in tumors: CDKN1B is on 12p13, which shows LOH in hematopoietic malignancies (7) and non-small cell lung cancer (8); CDKN1A is on 6p21, which shows LOH in ovarian carcinomas (13); and CDKN2C is on chromosome 1p32, which shows LOH in breast cancer (14). 1p32 also undergoes preferential LOH of the maternal allele in advanced neu.

CDKN1A

![Diagram of CDKN1A](image)

CDKN1B

![Diagram of CDKN1B](image)

CDKN2C

![Diagram of CDKN2C](image)
LACK OF IMPRINTING OF THREE HUMAN CKI GENES

**CDKN1A**

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Fig. 2. RT-PCR analysis of CDKN1A, CDKN1B, and CDKNC2 expression. Amplified cDNA or genomic DNA products derived from the indicated fetal tissues or adult colon were restricted with the appropriate enzyme as detailed in “Materials and Methods.”

- **F-1**: kidney; **F-2**: heart; **A-1**: intestine; **A-2**: lung; **A-3**: placenta; **col**: colon; **F**: fetus; **A**: adult. The two DNA bands of the CDKNC2 b allele are nearly identical in size (165 and 187 bp) and could not be resolved by electrophoresis. The genomic DNA products are larger than the cDNA products, but are aligned for clarity.

Although there are likely to be imprinted genes on at least 1p32, these CKIs do not appear to be imprinted. Thus, genomic imprinting is unlikely to contribute to the role of these genes in tumor progression.

These results do not exclude the possibility that other CKIs are imprinted. Alternatively, epigenetic silencing of these genes may not involve imprinting. For example, CDKN2A (p16) may undergo epigenetic silencing in tumors because some show absence of expression and hypermethylation of this gene (15, 16). However, it is important to note that alterations of methylation could be secondary to gene regulation and influenced by trans-acting factors. Furthermore, mice harboring a CDKN2A heterozygous knockout showed no decreased survival or abnormal phenotype, whereas homozygous knockout mice showed an increased incidence of malignancy (17). If CDKN2A were imprinted in mice, one would expect the same phenotype in half of the independently derived heterozygotes as in the homozygous knockout animals. Similarly, lack of imprinting of CDKN1B in mice is suggested by gene disruption experiments, which do not result in the generation of affected heterozygotes (18, 19).

The data reported here exclude imprinting of the specific genes studied, which are of considerable interest to human cancer genetics. They are also consistent with the observation that imprinted genes tend to cluster within defined chromosomal domains or may involve genes within the same signaling pathway, such as IGF2 and IGF2R, rather than involving separate genes of the same functional class.

Finally, the experiments reported here may serve as a useful paradigm for rapid investigation of imprinting performed concurrently with mutational analysis. As new candidate genes for cancer are identified and the tumors are scanned for mutation by, for example, single-strand conformational polymorphism-PCR, polymorphisms that are identified incidentally can easily be exploited to determine whether the gene is imprinted or not by RT-PCR analysis of the paired normal tissues.

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**REFERENCES**

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