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Advances in Brief

Cyclin-dependent Kinase Inhibitor $p57^{KIP2}$ in Soft Tissue Sarcomas and Wilms' Tumors[1]

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Abstract

Mammalian cyclin-dependent kinase inhibitors fall into two families, the INK4 and the CIP/KIP. The CIP/KIP family comprises three structurally related members, including $p21^{CiP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$. These proteins are all capable of inhibiting the progression of the cell cycle by binding and inhibiting G_1 cyclin/cyclin-dependent kinase complexes. In humans, $p57^{KIP2}$ is expressed specifically in skeletal muscle, heart, brain, kidney, and lung. Human *KIP2* resides in 11p15.5, a chromosomal region that is a common site for loss of heterozygosity in certain sarcomas, Wilms' tumors, and tumors associated with the Beckwith-Wiedemann syndrome. Because of the function, selective expression, and chromosomal location of $p57^{KIP2}$, we undertook the present study to search for potential mutations of *KIP2* in a cohort of 126 tumors composed of 75 soft tissue sarcomas and 51 Wilms' tumors. The *KIP2* gene was characterized by Southern blot, comparative multiplex PCR, PCR-single-strand conformational polymorphism, and DNA sequencing assays in these neoplasms. Deletions of the *KIP2* gene or point mutations at the region encoding the cyclin-dependent kinase inhibitory domain were not found in the tumors analyzed. The absence of *KIP2* mutations might indicate that these tumors arise due to defects at a closely linked but separate locus. Alternatively, similarly to the mouse homologue, inactivation of *KIP2* could occur via genomic imprinting.

Introduction

The CIP/KIP family of cyclin-dependent kinase inhibitors comprises three structurally related members, the $p21^{CIP1/WAF1}$ (1, 2), the $p27^{KIP1}$ (3, 4), and the recently cloned and characterized $p57^{KIP2}$ (5, 6). $p21^{CIP1/WAF1}$ was the first mammalian cyclin-dependent kinase inhibitor described which can bind and inhibit G_1 cyclin/Cdk[3] complexes as well as proliferating cell nuclear antigen (7). $p21^{CIP1/WAF1}$ is transcriptionally activated by p53, but in quiescent p53-deficient cells $p21^{CiP1}$ has been shown to be induced by serum or certain growth factors (8). $p27^{KiP1}$ is a negative regulator that participates in G_1 arrest induction by transforming growth factor-β, cell to cell contact, rapamycin, and agents that elevate cyclic AMP (3, 9). The $p57^{KIP2}$ protein sequence has four distinct domains, among them a Cdk inhibitory domain which is structurally similar to that of p21 (36% identity) and p27 (47% identity), a proline-rich region, an acidic domain, and a putative COOH-terminal nuclear localization site (5, 6). *In vitro* studies indicate that $p57^{KIP2}$ alone is a potent inhibitor of G_1 and S-phase Cdks. In contrast to the ubiquitous expression patterns of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, $p57^{KIP2}$ is expressed at high levels in specific embryonic and adult tissues such as skeletal muscle, heart, kidney, lung, and brain (5, 6).

The human *KIP2* maps to 11p15.5, a chromosomal region frequently deleted in distinct adult and childhood neoplasias, including rhabdomyosarcomas and Wilms' tumors (10–13). Within this chromosomal band, *KIP2* has been located between the *D11S601* and *D11S724* loci (14). Loss of the short arm of the chromosome 11 has also been reported in adult soft tissue sarcomas such as malignant fibrous histiocytomas (15). The chromosomal location, pattern of expression, and the antiproliferative function of $p57^{KIP2}$ raise the possibility that *KIP2* could be a tumor suppressor gene. To search for molecular abnormalities of *KIP2* in human cancer, we undertook the present study utilizing a large cohort of well-characterized clinical samples of adult sarcomas and pediatric neoplasms, including Wilms' tumors.

Materials and Methods

A total of 126 cases (75 soft tissue sarcomas and 51 Wilms' tumors) were analyzed. The soft tissue sarcomas studied included leiomyosarcomas (n = 12), liposarcomas (n = 17), fibrosarcomas (n = 5), malignant fibrous histiocytomas (n = 7), synovial sarcomas (n = 8), and peripheral nerve sheath tumors (n = 3). Three adult sarcomas were undifferentiated lesions. The pediatric tumors where represented by rhabdomyosarcomas (n = 18), Wilms' tumors (n = 51), and Ewing sarcomas (n = 2). Frozen tissue blocks were used as source for DNA extraction, using a nonorganic method (Oncor, Gaithersburg, MD). Normal DNA from the same patient was used as a control. Alterations in the *KIP2* gene were determined using Southern blot, comparative multiplex PCR, PCR-SSCP, and sequencing.

For the Southern blot analysis, a 0.7-kb cDNA fragment containing a partial human $p57^{KIP2}$ sequence was used as a probe to determine potential deletions or rearrangements. Membranes containing DNA digested with *Hin*dIII were prepared and hybridized as previously described (16). Evaluation of autographic band intensities was performed using an Ultrascan XL laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ), and a FUJIX Bio-Imaging Analyser (Fuji Photo Film Co., Japan). Relative amounts of *KIP2* present in each tumor were determined by comparing specific band signals with those obtained in the normal sample counterpart. For those cases where the amount of DNA was not sufficient to perform a Southern blot analysis, a comparative multiplex PCR was performed (17). The multiplex PCR consisted of a PCR reaction containing primers specific to the Cdk inhibitory domain of *KIP2* and primers for β-globin, which were used as internal controls. Primers used for *KIP2* were 5′-ATCCACGATGGAGCGTCTTGTC and 3′-CATGTC-CTGCTGGAAGTCGTAATC. Each reaction tube contained 100 ng DNA, 2.5 mM Mg^{2+}, 0.6 pmol of each *KIP2*-specific primer, 0.1 pmol of each β-globin primer, 80 mM deoxynucleotide triphosphate, 5% DMSO, 2.2 μCi [α-^{32}P]dCTP, 0.5 units Taq polymerase, and 1 × PCR buffer (Promega, Madison, WI) and was amplified for 20 cycles in a thermal cycler (Perkin Elmer/Cetus, Foster City, CA). The annealing temperature was 65°C (2 cycles), 62°C (2 cycles), and 60°C (14 cycles). The amplified products were resolved in a nondenaturing polyacrylamide gel, and the amount of *KIP2* present in each sample was assessed by comparison of *KIP2* and β-globin band

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; SSCP, single-strand conformational polymorphism.

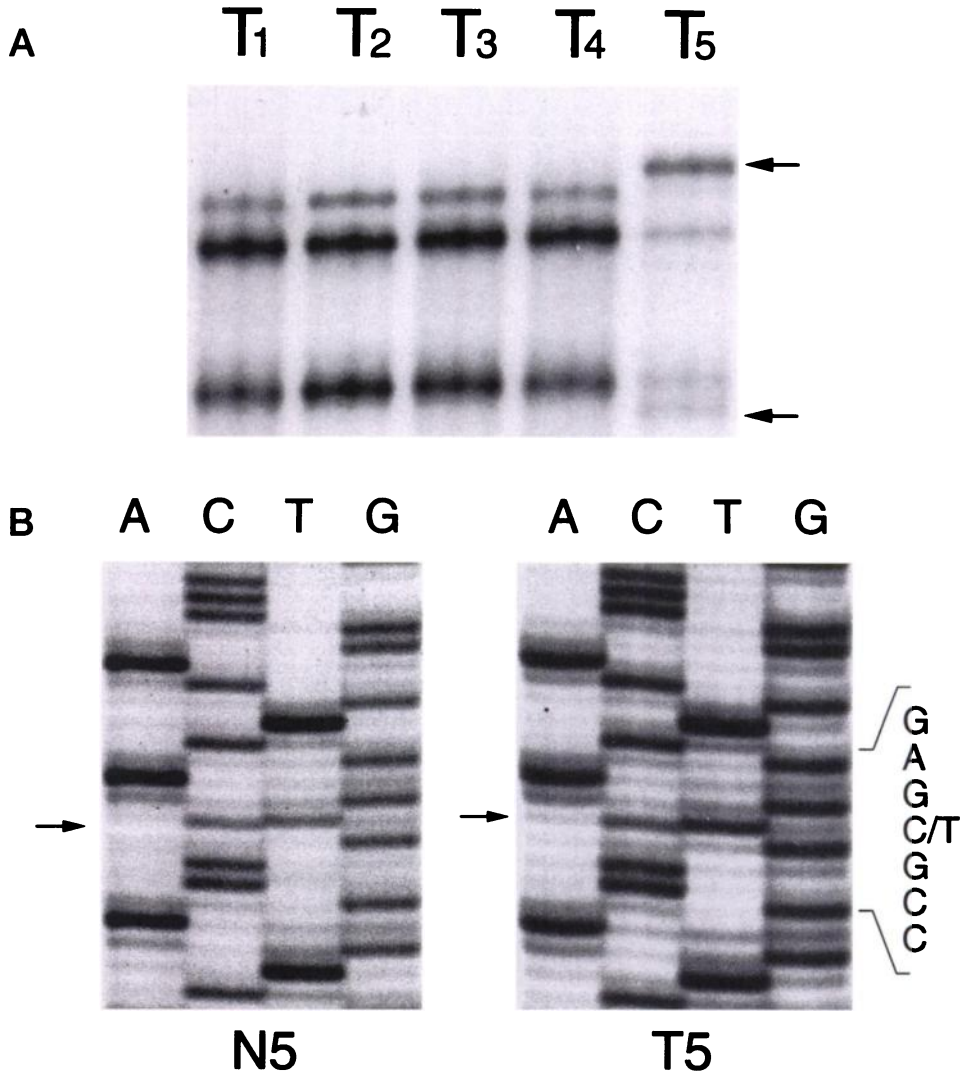

Fig. 1. *A*, This figure illustrates mobility shift in T5 detected by SSCP analysis of $p57^{KIP2}$. *B*, direct sequencing revealed that a base change, C to T substitution at codon 44, preserving a conserved arginine occurred, representing a polymorphism. *N*, normal; *T*, tumor.

signals in normal and tumor samples. All specimens were amplified in duplicate or triplicate in each assay. Samples that showed a relative decrease in signal were subjected to a new PCR reaction. We considered *KIP2* deleted if samples displayed <5% of the relative signal found in a set of normal DNAs.

For point mutation analysis, PCR-SSCP assays were performed according to a modification of the method reported by Orita *et al.* (18). The PCR amplifications were performed using the same primers and protocol conditions specified above; however, 30 cycles were used for the PCR reaction. The PCR products were denatured and loaded onto a nondenaturing 10% polyacrylamide gel that contained 10% glycerol. Electrophoresis was performed at room temperature for 24 to 36 h at 8 W. The gels were dried and exposed to X-ray film at −70°C. Specimens showing a differential band were purified (Geneclean; BIO 101 Inc., La Jolla, CA) and amplified for direct DNA sequencing. These amplifications were independent from those used for SSCP analysis. In some cases, due to a smaller amount of DNA available, specific fragments were cloned into a TA vector (Invitrogen Corporation, San Diego, CA) and sequenced using the dideoxynucleoside triphosphate method, with the Sequenase kit (United States Biochemical Corporation, Cleveland, OH). Five to 10 clones were selected for each amplified DNA fragment and individually sequenced.

Results and Discussion

Chromosome 11p15 deletions occur frequently in several types of human cancer, both sporadic and familial, suggesting that a tumor suppressor gene is present within the deleted chromosome region. The gene that codes for $p57^{KIP2}$ maps to 11p15.5 (6). This, along with the function and the selective expression of the human *KIP2*, indicates that alterations in the normal function of this gene could be involved in the development of certain neoplasms, including soft tissue sarcomas and Wilms' tumors (11, 12, 19).

In the present study, 75 cases of soft tissue sarcomas and 51 Wilms' tumors were analyzed using Southern blot with specific *KIP2* probes. No deletions or visible gene rearrangements were detected by comparing tumor *versus* normal DNA band signals. In a subgroup of soft tissue sarcoma cases, we performed a comparative multiplex PCR. Relative amounts of *KIP2* present in each tumor were determined by comparing specific band signals with those obtained with the control primers in the same PCR reaction. Control DNA samples were analyzed to establish the variability of the normal ratios. No deletions of $p57^{KIP2}$ were found in the DNA samples tested. The tumors analyzed contain a very small amount of normal cells, making it unlikely that the presence of the specific *KIP2* fragment observed using the comparative multiplex PCR is due to the presence of contaminating normal DNA.

To investigate the possibility of the presence of point mutations in the *KIP2* gene, all tumor cases were analyzed using PCR-SSCP. The area screened spanned the Cdk inhibitory region of $p57^{KIP2}$, which has been positioned on exon 1 (14). We reasoned that if *KIP2* was inactivated by point mutations, these might preferentially occur in the Cdk inhibitory region. A single sequence difference was detected, as

assessed by a shift in mobility, in one sample that corresponded to a liposarcoma (Fig. 1*A*). A new PCR was performed for this sample, and the purified DNA was directly sequenced. The nucleotide change at position 391 (C to T) encodes the same amino acid (Arg). Both normal and tumor DNA of this case contained the same base sequence (Fig. 1*B*), suggesting the detection of a polymorphism. We did not find point mutations in the genomic region analyzed for the cases studied.

Although the features of *$p57^{KIP2}$* suggest that it may act as a tumor suppressor gene, we did not observe *KIP2* deletions or point mutations in the Cdk-binding domain in the tumors screened. It is possible that in the neoplasms the 11p15 losses affect a closely linked but separate locus. Alternatively, *KIP2* could be inactivated by genomic imprinting. Several observations support this hypothesis. Some types of childhood cancers, including Wilms' tumor, adrenocortical carcinoma, hepatoblastoma, and rhabdomyosarcoma, display a specific loss of maternal 11p15 alleles, suggesting that genomic imprinting may play a role in these cancers (20–22). *KIP2* is located near a cluster of imprinted genes, including *IGF2* and *H19* (23). Hatada and Mukai (24) showed recently that a mouse homologue of *KIP2* is genomically imprinted. If the human *KIP2* is also subject to allele-specific methylation, a significant decrease in the amount of $p57^{KIP2}$ would be evident when the remaining (nonimprinted) allele is lost by deletion or rearrangement. Conversely, point mutations may exist in other exonic or intronic sites not analyzed in the present study.

Our results, along with the low rate or absence of detectable genetic abnormalities of the other members of the CIP/KIP family ($p21^{CIP1/WAF1}$ and $p27^{KIP1}$) suggest that if the inactivation of CIP/KIP members play a role in the uncoordinated cell growth of the tumor cells, it may be due to posttranscriptional or posttranslational modifications (25–29). Additional studies are required to address these critical issues.

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