Mutation and expression analysis of the cyclin-dependent kinase inhibitor gene p27/Kip1 in pituitary tumors

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Abstract

By regulating cyclin–cyclin-dependent kinase (CDK) complex activity, individual CDK inhibitors (CDKIs) are potential tumor suppressors. One of the CDKIs, p27/Kip1, binds to a variety of CDK–cyclin complexes. A link between loss of p27/Kip1 function and development of pituitary tumors was suggested by the formation of pituitary tumors in almost all mice with germline deletion of the p27/Kip1 gene. However, genetic aberrations in the p27/Kip1 locus have not been analyzed in human pituitary tumors. We investigated eighteen non-functioning and GH-secreting pituitary tumor samples for p27/Kip1 mutations by single-strand conformational polymorphism (SSCP) following PCR. We found five abnormally migrating samples on the PCR–SSCP analysis. The sequence of these samples revealed a polymorphism of codon 109 (Val→Gly), which has been previously described. No other structural changes of p27/Kip1 were found in these pituitary tumors within the coding region. In addition, no difference in p27/Kip1 protein levels in pituitary tumor tissues compared with normal pituitary tissues was demonstrated by immunostaining. These data suggest that both p27/Kip1 mutations and decreases in p27/Kip1 protein levels are infrequent in the development of pituitary tumors.


Introduction

In the past few years, understanding of the cell cycle has advanced rapidly (Reed 1992, Pines 1993, Sherr 1993). During cellular proliferation, cyclins and cyclin-dependent kinases (CDKs) play important roles in the cell cycle. Recently, a newly recognized family of proteins which inhibit CDKs has been identified. These cyclin-dependent kinase inhibitors (CDKIs) form two classes. The CDKN2B/INK4B/p15 (Hannon & Beach 1994), CDKN2/INK4A/p16 (Serrano et al. 1993), and p18/INK4C (Guan et al. 1994) genes form one class. The second class includes p21/WAF1, p27/Kip1 and p57 (El-Deiry et al. 1993, Gu et al. 1993, Harper et al. 1993, Hunter 1993, Xiong et al. 1993, Polyak et al. 1994, Toyoshima & Hunter 1994, Matsuoka et al. 1995). These homologous proteins bind to a variety of CDK–cyclin complexes. Transforming growth factor β (TGF-β) may induce cell growth arrest through p27/Kip1 activation. TGF-β often loses its ability to arrest growth of transformed cells; this could potentially occur through a defect in p27/Kip1. By regulating cyclin–CDK complex activity, individual CDKIs are potential tumor suppressors. Therefore, their inactivation might contribute to development of cancer. Indeed, mutations and deletions of CDKN2B/INK4B/p15 and CDKN2/INK4A/p16 are common in human malignancies (Kamb et al. 1994, Nobori et al. 1994, Hirama & Koeffler 1995, Takeuchi et al. 1995). However, despite an extensive search for molecular aberrations of p27/Kip1, no significant mutations of p27/Kip1 have been reported (Kawamata et al. 1995, Pietinen et al. 1995, Ponce-Castañeda et al. 1995, Seriu et al. 1996, Stegmaier et al. 1996, Takeuchi et al. 1996).

A link between loss of p27/Kip1 function and pituitary tumors was suggested because almost all mice having germline deletion of the p27/Kip1 gene developed intermediate lobe pituitary hyperplasia and pituitary tumors (Fero et al. 1996, Kiyokawa et al. 1996, Nakayama et al. 1996). However, alterations of the p27/Kip1 gene in human pituitary tumors have not been analyzed. In this report, we have investigated if mutations of the p27/Kip1 gene occur in human pituitary tumors, and have studied p27/Kip1 protein levels in different pituitary tumor types.

Materials and Methods

Mutational analysis of the p27/Kip1 gene

Eighteen DNAs from pituitary tumors were obtained at the time of surgery after informed consent from patients.
These tumors included ten non-functioning tumors, and eight growth hormone-producing tumors. The PCR amplification of each region was performed with the incorporation of [α-³²P]dCTP as described previously (Kawamata et al. 1995). Primers used were derived as reported previously (Kawamata et al. 1995). The products were separated in 0·5 × HydroLink MDE Gel (J T Baker, Inc., Phillipsburg, NJ, USA) at room temperature. The gels were dried and exposed to X-ray films at −80°C overnight. The PCR products were directly sequenced by the cycle sequence method (GIBCO-BRL, Rockville, MD, USA).

**Immunostaining for p27/Kip1**

Tissue samples from 18 other pituitary tumors were obtained after trans-sphenoidal resection and included prolactinomas (5 cases), somatotroph tumors (4 cases), corticotroph tumors (4 cases) and non-functioning tumors (5 cases). Normal tissues were obtained after trans-sphenoidal resection and included sections of carcinomas of bladder which were either positive or negative for p27/Kip1. These controls included sections of carcinomas of bladder which were either positive or negative for p27/Kip1. These tissues, including controls, were fixed in 10% buffered formalin, embedded in paraffin and 4-microns sections were placed on poly-L-lysine coated slides. Sections stained with hematoxylin and eosin were used for histologic diagnosis. Immunoperoxidase staining of the paraffin sections was performed using the avidin–biotin complex (ABC) method (Vector, Burlingame, CA, USA) as described by the manufacturer with aminoethylcarbazole or diaminobenzidine as the chromogen. After routine deparaffinization, rehydration and blockade of endogenous peroxidase activity, antigen retrieval was performed as previously described (Shi et al. 1995). The primary antibodies used to establish the type of pituitary tumor included polyclonal antiserum against adrenocorticotropic hormone, follicle-stimulating hormone, growth hormone (GH), and prolactin (Signet Laboratories, Dedham, MA, USA). p27/Kip1 monoclonal antibody (Lab Vision Corporation, Fremont CA, USA) was used at a 1:50 dilution. Controls included appropriate positive and negative tissues as well as slides with ‘no primary antibody’. After incubation with the primary antibody, staining was completed using an automated stainer (Techmate 1000: Bio Tek, Santa Barbara, CA, USA). A very light hematoxylin counterstain was used.

**Results**

To look for point mutations by PCR-single-strand conformational polymorphism (SSCP), the coding region of the p27/Kip1 gene was divided into three regions: Ia, Ib (both in exon 1), and II (in exon 2). For regions Ia and II, no shifted bands were detectable in the pituitary tumor samples. For region Ib of exon 1, three patterns of bands occurred (Fig. 1). Sequence analysis showed that the shifted bands were due to a previously identified polymorphism at codon 109 (GTC→GGC; Val→Gly). The rate of homozygosity of allele A was 72% (13 of 18), homozygosity of allele B was 11% (2 of 18), and heterozygosity was 17% (3 of 18). No other mutations of p27/Kip1 were found in these pituitary tumors within the coding region.

Immunoperoxidase staining for p27/Kip1 antigen in normal pituitary tissues obtained at autopsy and from normal glands adjacent to microadenomas showed nuclear immunoreactivity throughout the anterior pituitary (Fig. 2B). Positive staining was found in many cells but varied in intensity from weak to very strong. Comparison with adjacent sections stained for pituitary hormones showed that the labeling was not confined to any specific cell type. All pituitary tumor types studied showed tumor cells with nuclear positivity for p27/Kip1 (Fig. 2C–F). In each case, the tumor was composed of a population of strongly positive, weekly positive and negative cells. No relationship was found between the type of tumor and the extent of p27/Kip1 reactivity. Controls included sections of carcinomas of bladder which were either positive or negative for p27/Kip1, and slides in which the primary antibody was omitted (Fig. 2A). Minimal nonspecific immunoreactivity was found.

**Discussion**

The CDKIs can be categorized based on their structure. One group includes the CDKN2B/INK4B/p15, CDKN2/INK4A/p16, and p18/INK4C. The CDKN2B/INK4B/p15 and CDKN2/INK4A/p16 genes are very
frequently mutated in a variety of cancers (Kamb et al. 1994, Nobori et al. 1994, Hirama & Koeffler 1995, Takeuchi et al. 1995). The p27/Kip1 and p21/WAF1 belong to the other group. In this study, no structural alterations of the p27/Kip1 gene were found in pituitary tumors. We and others have analyzed many kinds of human malignancies for alterations of the p27/Kip1 gene. We identified one nonsense mutation in a breast cancer sample and another nonsense mutation in a sample of adult T cell leukemia (ATL) (Morosetti et al. 1995, Spirin et al. 1994).
Homozygous deletion of the \( p27/Kip1 \) gene was found in one case of B cell non-Hodgkin’s lymphoma and one case of ATL (Morosetti et al. 1995). No abnormalities were seen in a total of 573 samples of bladder, prostate, renal, pancreas, lung, ovarian, testicular, endometrial, gastric, and cervical cancers as well as samples of germ cell tumor, melanoma, sarcoma, acute lymphoblastic leukemia, and acute myelogenous leukemia (Kawamata et al. 1995, Seriu et al. 1996, Stegmaier et al. 1996, Takeuchi et al. 1996). Taken together, abnormalities of the \( p27/Kip1 \) gene are rarely found in human malignancies. We reported previously that abnormalities of the \( p21/WAF1 \) gene were very rare (Shiohara et al. 1994). Thus the latter group of the CDKIs, including \( p27/Kip1 \) and \( p21/WAF1 \), are rarely mutated in human malignancies.

In this study, we identified the previously described polymorphism of codon 109 (GTC→GCC; Val→Gly) of the \( p27/Kip1 \) gene. The rate of homozygosity of allele A was 72% (13 of 18), homozygosity of allele B was 11% (2 of 18), and heterozygosity was 17% (3 of 18) in this study. These frequencies were compatible with those found in our previous study analyzing a variety of 432 human cancers (A/A 72%, B/B 11%, and A/B 4%) (Kawamata et al. 1995).

Recent studies have shown that the expression levels of \( p27/Kip1 \) are decreased in aggressive breast and colorectal cancers and that enhanced degradation can be a mechanism to eliminate \( p27/Kip1 \) in tumors (Catzavelos et al. 1994). These frequencies were compatible with those found in our previous study analyzing a variety of 432 human cancers (A/A 72%, B/B 11%, and A/B 4%) (Kawamata et al. 1995).

Mutation of the \( p27/Kip1 \) gene is a rare event in non-functioning and GH-secreting pituitary tumors as shown by this study. Could the low frequency of detectable \( p27/Kip1 \) mutations reflect false negative results? Technical problems in the detection of \( p27/Kip1 \) mutations are unlikely; we found \( p27/Kip1 \) confirming that \( p27/Kip1 \) are decreased in aggressive breast and colorectal tumors. This could be explained by the fact that pituitary tumors have a low level of cell proliferation.

Mutation of the \( p27/Kip1 \) gene is a rare event in non-functioning and GH-secreting pituitary tumors as shown by this study. Could the low frequency of detectable \( p27/Kip1 \) mutations reflect false negative results? Technical problems in the detection of \( p27/Kip1 \) mutations are unlikely; we found \( p27/Kip1 \) mutations in breast cancer and in ATL samples using exactly the same method (Morosetti et al. 1995, Spirin et al. 1996). However, more samples will be necessary to determine the real frequency of \( p27/Kip1 \) mutations in pituitary tumors. Moreover, our results show no decrease in \( p27/Kip1 \) protein levels in pituitary tumors. This could be explained by the fact that pituitary tumors have a low level of cell proliferation.

Several molecular alterations have been described in pituitary tumors (Shimon & Melmed 1997). Loss of the \( p16 \) gene product occurred at a high frequency (Woloschak et al. 1996). Although the \( RB \) gene itself is not an important target in pituitary tumorigenesis, alteration in a locus close to \( RB \) is likely to be important in a subset of aggressive benign pituitary tumors (Pei et al. 1995). No mutations were found in \( p53 \), \( H-ras \), \( K-ras \), and \( N-ras \) genes in pituitary tumors (Herman et al. 1993, Levy et al. 1994). However, \( H-ras \) mutations were detected when pituitary carcinoma metastasized (Pei et al. 1994). Thrytropin-releasing hormone receptor structure was normal in pituitary tumors (Faccenda et al. 1996). The \( nm23 \) gene expression was reduced in invasive pituitary tumors (Takino et al. 1995).

In summary, although deletion of the \( p27/Kip1 \) gene in mice leads to pituitary tumors, pituitary tumors in humans do not exhibit \( p27/Kip1 \) gene mutations or a decrease in \( p27/Kip1 \) protein levels.

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