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

S Takeuchi, H P Koeffler, D R Hinton¹, I Miyoshi², S Melmed and I Shimon

Department of Medicine, Cedars-Sinai Research Institute, UCLA School of Medicine, Los Angeles, California 90048, USA, ¹Department of Pathology, University of Southern California School of Medicine, Los Angeles, California 90033, USA and ²Department of Internal Medicine, Kochi Medical School, Kochi, Japan (Requests for offprints should be addressed to S Takeuchi, 3rd Department of Medicine, Kochi Medical School, Okohcho, Nankoku, Kochi 783, Japan)

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.

Journal of Endocrinology (1998) **157**, 337–341

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, Pietenpol *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 - ^{32} P]dCTP as described previously (Kawamata *et al.* 1995). Primers used were derived as reported previously (Kawamata *et al.* 1995). The products were separated in $0.5 \times$ 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 from autopsy (1 case), and from incidental removal of the anterior pituitary (2 cases) in patients with adjacent microadenomas. 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 aminoethylcarbizole 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 antisera against adrenocorticotrophic hormone, follicle-stimulating hormone, thyroid-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

Figure 1 PCR-SSCP analysis of *p27/Kip1* gene exon 1b in pituitary tumors. Three electrophoretic patterns were identified by SSCP from the pituitary tumor samples. Sample no. 2 shows homozygosity for allele B; sample no. 3 demonstrates heterozygosity for the A and B alleles; sample no. 4 shows homozygosity for allele A.

(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, weakly 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

Figure 2 Photomicrographs of human pituitary tumors stained by the immunoperoxidase (ABC) method for p27/Kip1 protein. In A, a 'no primary antibody' control (same tumor as E) lightly counterstained with hematoxylin shows only the nuclear staining of the counterstain. In B, a normal pituitary section shows nuclear immunoreactivity for p27/Kip1. The degree of staining varied from cell to cell; however some cells were strongly stained (arrow). Nuclear immunoreactivity was found in all tumors studied including prolactinomas (C), somatotroph tumors (D), corticotroph tumors (E), and non-functioning tumors (F).

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.*

1996). 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→GGC; 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.* 1997, Loda *et al.* 1997, Porter *et al.* 1997). 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.

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 difference in *p27/Kip1* immunoreactive protein levels in different pituitary tumor types, thus confirming that *p27/Kip1* mutations have no significant role in human pituitary pathogenesis.

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-*, *K-*, 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). Thyrotropin-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.

Acknowledgements

This work was supported in part by the National Institutes of Health Grants, the Concern Foundation, and Parker Hughes Trust. H PK is a member of the Jonsson Comprehensive Cancer Center and holds the endowed Mark Goodson Chair of Oncology Research at Cedars-Sinai Medical Center/UCLA School of Medicine.

References

- Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, Shaw P, Yeger H, Morava-Protzner I, Kapusta L, Franssen E, Pritchard KI & Slingerland JM 1997 Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. *Nature Medicine* **3** 227–230.
- El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW & Vogelstein B 1993 WAF1, a potential mediator of p53 tumor suppression. *Cell* **75** 817–825.
- Faccenda E, Melmed S, Bevan JS & Eidne KA 1996 Structure of the thyrotropin-releasing hormone receptor in human pituitary adenomas. *Clinical Endocrinology* **44** 341–347.
- Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai L-H, Broudy V, Perlmutter RM, Kaushansky K & Roberts JM 1996 A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27^{Kip1}-deficient mice. *Cell* **85** 733–744.
- Gu Y, Turk CW & Morgan DO 1993 Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. *Nature* **366** 707–710.
- Guan K-L, Jenkins CW, Li Y, Nichols MA, Wu X, O'Keefe CL, Matera G & Xiong Y 1994 Growth suppression by p18, a p16^{INK4A/MTS1}- and p14^{INK4B/MTS2}- related CDK6 inhibitor, correlates with wild-type pRb function. *Genes and Development* **8** 2939–2952.
- Hannon GJ & Beach D 1994 p15^{INK4B} is a potential effector of TGF-β-induced cell cycle arrest. *Nature* **371** 257–261.
- Harper JW, Adami GR, Wei N, Keyomarsi K & Elledge SJ 1993 The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75** 805–816.
- Herman V, Drazin NZ, Gonsky R & Melmed S 1993 Molecular screening of pituitary adenomas for gene mutations and rearrangements. *Journal of Clinical Endocrinology and Metabolism* **77** 50–55.
- Hirama T & Koeffler HP 1995 Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood* **86** 841–854.
- Hunter T 1993 Braking the cycle. *Cell* **75** 839–841.
- Kamb A, Gruis NA, Weaver-Feldhaus J, Lin Q, Harshman K, Tavtigian SV, Stockert E, Day III RS, Johnson BE & Skolnick MH 1994 A cell cycle regulator potentially involved in genesis of many tumor types. *Science* **264** 436–440.

- Kawamata N, Morosetti R, Miller CW, Park D, Spirin KS, Nakamaki T, Takeuchi S, Hatta Y, Simpson J, Wilczynski S, Lee YY, Bartram CR & Koeffler HP 1995 Molecular analysis of the cyclin-dependent kinase inhibitor p27/kip1 gene in human malignancies. *Cancer Research* **55** 2266–2269.
- Kiyokawa H, Kineman RD, Manova-Todorova KO, Soares VC, Hoffman ES, Ono M, Khanam D, Hayday AC, Frohman LA & Koff A 1996 Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27^{Kip1}. *Cell* **85** 721–732.
- Levy A, Hall L, Yeudall WA & Lightman SL 1994 p53 gene mutations in pituitary adenomas: rare events. *Clinical Endocrinology* **41** 809–814.
- Loda M, Cukor B, Tam SW, Lavin P, Fiorentino M, Draetta GF, Jessup JM & Pagano M 1997 Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nature Medicine* **3** 231–234.
- Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW & Elledge SJ 1995 p57^{KIP2}, a structurally distinct member of the p21^{CIP1} Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes and Development* **9** 650–662.
- Morosetti R, Kawamata N, Gombart AF, Miller CW, Hatta Y, Hiramata T, Said JW, Tomonaga M & Koeffler HP 1995 Alterations of p27^{KIP1} gene in non-Hodgkin's lymphomas and adult T-cell leukemia/lymphoma. *Blood* **86** 1924–1930.
- Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY & Nakayama K 1996 Mice lacking p27^{Kip1} display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85** 707–720.
- Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K & Carson AD 1994 Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368** 753–756.
- Pei L, Melmed S, Scheithauer B, Kovacs K & Prager D 1994 H-ras mutations in human pituitary carcinoma metastases. *Journal of Clinical Endocrinology and Metabolism* **78** 842–846.
- Pei L, Melmed S, Scheithauer B, Kovacs K, Benedict WF & Prager D 1995 Frequent loss of heterozygosity at the retinoblastoma susceptibility gene (*RB*) locus in aggressive pituitary tumors: evidence for a chromosome 13 tumor suppressor gene other than *RB*. *Cancer Research* **55** 1613–1616.
- Pietenpol JA, Bohlander SK, Sato Y, Papadopoulos N, Lin B, Friedman C, Trask BJ, Roberts JM, Kinzler KW, Rowley JD & Vogelstein B 1995 Assignment of the human p27^{kip1} gene to 12p13 and its analysis in leukemia. *Cancer Research* **55** 1206–1210.
- Pines J 1993 Cyclins and cyclin-dependent kinases; take your partners. *Trends in Biochemical Sciences* **18** 195–197.
- Polyak K, Lee M-H, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P & Massague J 1994 Cloning of p27^{KIP1}, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78** 59–66.
- Ponce-Castañeda MV, Lee MH, Latres E, Polyak K, Lacombe L, Montgomery K, Mathew S, Krauter K, Sheinfeld J, Massague J & Cordon-Cardo C 1995 p27^{KIP1}: chromosomal mapping to 12p12–12p13.1 and absence of mutations in human tumors. *Cancer Research* **55** 1211–1214.
- Porter P, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, Daling JR & Roberts JM 1997 Expression of cell-cycle regulation of p27kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nature Medicine* **3** 222–225.
- Reed SI 1992 The role of p34 kinases in the G1 to S-phase transition. *Annual Review of Cell Biology* **8** 529–561.
- Seriu T, Erz D & Bartram CR 1996 Germline configuration of the p27^{KIP1} gene in childhood acute lymphoblastic leukemia (ALL). *Leukemia* **10** 345.
- Serrano M, Hannon GJ & Beach D 1993 A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK 4. *Nature* **366** 704–707.
- Sherr CJ 1993 Mammalian G1 cyclins. *Cell* **73** 1059–1065.
- Shi S-R, Imam A, Young L, Cote R & Taylor CR 1995 Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *Journal of Histochemistry and Cytochemistry* **43** 193–201.
- Shimon I & Melmed S 1997 Genetic basis of endocrine disease: pituitary tumor pathogenesis. *Journal of Clinical Endocrinology and Metabolism* **82** 1675–1681.
- Shiohara M, El-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen DL, Vogelstein B & Koeffler HP 1994 Absence of WAF1 mutations in a variety of human malignancies. *Blood* **84** 3781–3784.
- Spirin KS, Simpson JF, Takeuchi S, Kawamata N, Miller CW & Koeffler HP 1996 p27/Kip1 mutation found in breast cancer. *Cancer Research* **56** 2400–2404.
- Stegmaier K, Takeuchi S, Golub TR, Bohlander SK, Bartram CR, Koeffler HP & Gilliland DG 1996 Mutational analysis of the candidate tumor suppressor genes *TEL* and *KIP1* in childhood acute lymphoblastic leukemia. *Cancer Research* **56** 1413–1417.
- Takeuchi S, Bartram CR, Seriu T, Miller CW, Tobler A, Janssen JWG, Reiter A, Ludwig W-D, Zimmermann M, Schwaller J, Lee E, Miyoshi I & Koeffler HP 1995 Analysis of a family of cyclin-dependent kinase inhibitors: p15/MTS2/INK4B, p16/MTS1/INK4A and p18 genes in acute lymphoblastic leukemia of childhood. *Blood* **86** 755–760.
- Takeuchi S, Mori N, Koike M, Slater J, Park S, Miller CW, Miyoshi I & Koeffler HP 1996 Frequent loss of heterozygosity in region of the KIP1 locus in non-small cell lung cancer: evidence for a new tumor suppressor gene on the short arm of chromosome 12. *Cancer Research* **56** 738–740.
- Takino H, Herman V, Weiss M & Melmed S 1995 Purine-binding factor (nm23) gene expression in pituitary tumors: marker of adenoma invasiveness. *Journal of Clinical Endocrinology and Metabolism* **80** 1733–1738.
- Toyoshima H & Hunter T 1994 p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. *Cell* **78** 67–74.
- Woloschak M, Yu A, Xiao J & Post KD 1996 Frequent loss of the p16^{INK4a} gene product in human pituitary tumors. *Cancer Research* **56** 2493–2496.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R & Beach D 1993 p21 is a universal inhibitor of cyclin kinases. *Nature* **366** 701–703.

Received 29 September 1997

Accepted 11 December 1997