RAPID COMMUNICATION Jpn. J. Cancer Res. 88, 229–233, March 1997

# Concomitant Presence of p16/Cyclin-dependent Kinase 4 and Cyclin D/Cyclin-dependent Kinase 4 Complexes in LNCaP Prostatic Cancer Cell Line

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**The cyclin D/cyclin-dependent kinase (CDK)/CDK-inhibitory proteins/retinoblastoma protein (pRb) pathway is hypothesized to control the G1-S check point. The role of this pathway is reported to be different depending on the status of pRb. In the present study, we examined nine human urological tumor cell lines. Cells lacking functional pRb expressed p16, instead of forming cyclin D/CDK4 complex. In the LNCaP prostatic cancer cell line, however, both p16/CDK4 and cyclin D/CDK4 complexes were present independently, probably because of partial loss of pRb. In view of the concomitant presence of the incompatible complexes, LNCaP should provide us with a valuable model for the study of this pathway in cancer cells.**

Key words: Cyclin-dependent kinase 4 — p16 — Retinoblastoma protein — LNCaP

Restriction point (G1-S transition in mammalian cells) control is considered to be one of the most common targets of tumorigenesis.[1] Many lines of evidence suggest that the cyclin D/cyclin-dependent kinase (CDK)/CDK-inhibitory proteins (CKI)/retinoblastoma protein (pRb) pathway is the major regulator of this point.[2,3] There are two hypotheses concerning this pathway; one is that pRb is downstream of cyclin D,[4,5] and the other is that cyclin D is negatively regulated by pRb.[6] However, the mechanism of restriction point control is different in RB-positive and RB-negative cells. In the presence of functional pRb, CDK4, the major G1 cyclin kinase, requires cyclin D association for its activation and controls cell cycle progression through pRb phosphorylation.[7] Conversely, some recent studies have revealed cyclin D dissociation from CDK4 and loss of the cyclin D requirement for cell cycle transition in the absence of functional pRb.[5,8] One of the CKI, *p16*/multiple tumor suppressor gene 1 (MTS1), is a putative tumor-suppressor because p16 arrests cell proliferation at G1 by inhibiting CDK4/cyclin D activity,[7] and is frequently inactivated in a large variety of human cancers[9] and in p16-deficiency-facilitated tumor development in p16/MTS1 knock-out mice.[10] As p16 did not function as a tumor suppressor in cells lacking functional pRb,[2] p16 may buffer the cyclin D/CDK/CKI/pRb pathway by preventing premature pRb phosphorylation in cells that retain pRb activity.[7] We are interested in the implication of the tumor suppressor genes relevant to restriction point control in tumorigenesis of urological cancers and we have reported genetic alterations of some of them, including p53 and p16.[11,12] In this study, we extended these analyses to the protein level in urological tumor cell lines.

To examine the mechanism of restriction point control in urological tumor cell lines, we first analyzed by immunoblotting the protein levels of CDK4, CDK2, three D-type cyclins, proliferating cell nuclear antigen (PCNA), pRb and p16 in nine human urological tumor cell lines (one embryonic kidney, three renal cell carcinomas, three prostatic cancers and two bladder cancers) (Fig. 1). All cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum. Exponentially growing cells were trypsinized, harvested by centrifugation and lysed in NP-40 buffer (50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 0.5% NP-40, 10 $\mu$g/ml of leupeptin, and 10 $\mu$g/ml of aprotinin). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in polyacrylamide gels of appropriate concentrations. After incubation of the transferred membranes with each antibody (cyclin D1 (HD11), D2 (C-17), D3 (C-16), CDK4 (H-22), CDK2(M2), PCNA (PC10)(Santa-Cruz Biotechnology, Inc.), p16 (15126E) (Pharmingen, San Diego, CA), pRb (3H9)(MBL)), the reactive bands were detected using peroxidase-labeled antibodies and visualized by enhanced chemical luminescence. Table I summarizes the data on the immunoblotting of whole cell lysates and immunoprecipitates with the anti-CDK4 antibody. The expression of CDK4 and CDK2 was detected in all cell lines. Although the three D-type cyclins were differentially expressed in various cell lineages,[13] we could not find any characteristic ex-

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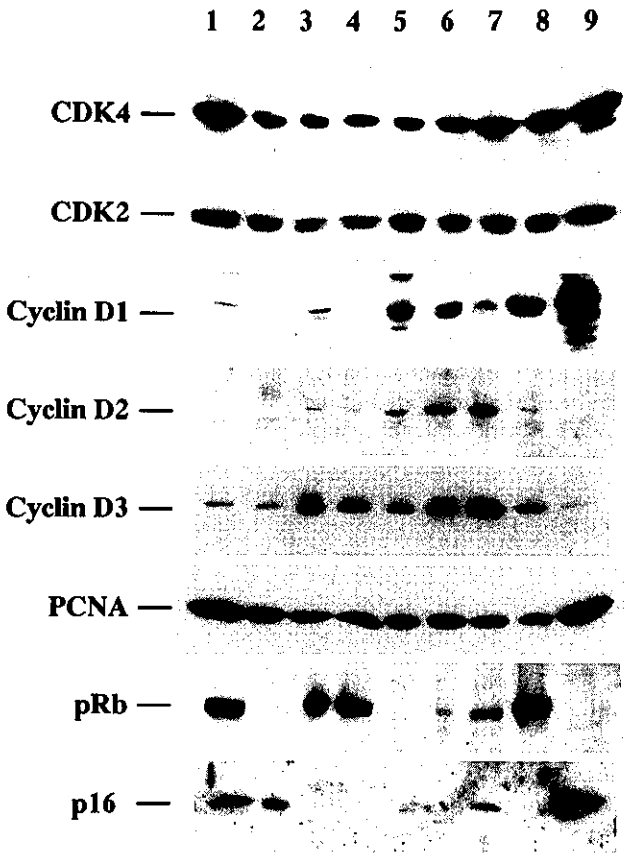


Fig. 1. Expressions of cyclin-dependent kinase (CDK)4, CDK2, D-type cyclins, retinoblastoma protein (pRb) and p16 in nine human urological tumor cell lines evaluated by western blot analyses. Lanes, (1) embryonic kidney cell line 293, (2) renal cell carcinoma cell line NC65, (3) Caki-1, (4) ACHN, (5) prostatic cancer cell line DU145, (6) PC-3, (7) LNCaP, (8) bladder cancer cell line T24 and (9) 5637. The amount of applied protein per lane was adjusted to 30 $\mu$g.

pression of the three D-type cyclins according to the cell type of the urological tumor cell lines. The expression of PCNA was taken as evidence of cell proliferation. Our antibody did not detect the mutant small-sized pRb of the DU145 cell line that was caused by a deletion in exon 21 of RB,[14] and showed weaker reactivity with pRb of the PC-3 cell line than previously reported.[14] The protein expression of p16 was compatible with our previous findings in the genetic study of renal cell carcinoma cell lines.[12] pRb expression of the renal cell carcinoma cell lines harboring genetic alteration of p16/MTS1[12] was preserved. Embryonic kidney cell line 293, in which the pRb was functionally inactivated by E1A, expressed p16. Although very low levels of protein might not be detected by our antibodies, the expression of p16 did not accompany functionally active pRb except for a prostatic cancer cell line LNCaP.

As CDK4 required the association of D-type cyclins for its activation, we next examined the formation of cyclin Ds/CDK4 and p16/CDK4 complexes. We examined their association by western blot analysis, using anti-cyclin Ds and p16 antibodies, of immunoprecipitates with anti-CDK4 antibody (Fig. 2A). For immunoprecipitation, cell lysates were cleared, incubated with 1 $\mu$g of the antibody and precipitated with protein A-Sepharose beads. Our findings suggest that CDK4 is sequestered from cyclin D by its association with p16, except in the LNCaP cell line. Although the frequency of genetic alteration of p16/MTS1 in tumor cell lines was high in previous reports,[9, 15] p16 of all cell lines that were recognized by our antibody seemed normal, judging from its electrophoretic mobility and the ability to form a complex with CDK4 *in vivo*. In contrast, the anti-CDK4 antibody immunoprecipitated three cyclin Ds and p16 in LNCaP; this was confirmed by western blot analysis, using the anti-CDK4 antibody, of the immunoprecipitates with anti-cyclin D and -p16 antibodies of the LNCaP cell lysate (Fig. 2B). Then, we examined whether cyclin D, CDK4 and p16 formed a ternary complex by means of western blot analysis, using the anti-cyclin D3 antibody, of the immunoprecipitate with the anti-p16 antibody of the LNCaP cell lysate (Fig. 2C). The anti-p16 antibody did not immunoprecipitate cyclin D3 in LNCaP. The use of a different lysis buffer (50 m*M* Hepes, 150 m*M* NaCl, 0.1% Tween 20, 10 $\mu$g/ml of leupeptin, 10 $\mu$g/ml of aprotinin) gave similar results. These findings suggest that some part of CDK4 assembles with cyclin D and another part with p16, independently.

Although Geradts *et al.* reported that both pRb and p16 could be detected in 43% of all carcinomas examined by immunohistochemical assay,[16] p16 expression detected by immunoblotting did not accompany functionally active pRb in several studies, including the present one.[7, 17, 18] The silencing of one tumor suppressor, pRb, accompanied by the overexpression of another, p16, might be caused by a negative feedback loop by which pRb transcriptionally represses p16 expression.[19] Hara *et al.* very recently showed that p16 transcription was affected by the status of pRb.[20] Although cyclin D/CDK4/p16 ternary complex and independent binary complexes of cyclin D/CDK4 and p16/CDK4 were formed *in vitro*,[21, 22] the p16/CDK4 complex has been considered to assemble at the expense of the cyclin D/CDK4 complex *in vivo*.[7, 22] Indeed, DNA tumor virus oncoproteins that could inactivate pRb did not disrupt cyclin D1/CDK4 complexes in human cells lacking p16.[22] In prostatic cancer cell line LNCaP, however, we found the expression of both cyclin D and p16 by im-

Table I. Expression of Restriction Point-controlling Molecules and Their Association with CDK4 in a Series of Urological Tumor Cell Lines

| Cell line origin | 293[a] kidney | NC65 kidney | Caki-1 kidney | ACHN kidney | DU145 prostate | PC-3 prostate | LNCaP prostate | T24 bladder | 5637 bladder |
|---|---|---|---|---|---|---|---|---|---|
| Whole cell lysates | | | | | | | | | |
| CDK4 | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ |
| CDK2 | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ |
| cyclin D1 | + | − | + | ± | 2+ | 2+ | + | 2+ | 2+ |
| cyclin D2 | − | − | + | ± | + | 2+ | 2+ | + | − |
| cyclin D3 | + | + | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | + |
| PCNA | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ |
| pRb | 2+ | − | 2+ | 2+ | −[b] | ± | + | 2+ | − |
| p16 | 2+ | 2+ | − | − | − | − | + | − | 2+ |
| Immunoprecipitates with anti-CDK4 antibody | | | | | | | | | |
| cyclin D1 | − | − | + | + | + | + | 2+ | + | − |
| cyclin D2 | − | − | 2+ | 2+ | + | + | 2+ | + | − |
| cyclin D3 | ± | − | + | 2+ | ± | + | 2+ | ± | − |
| p16 | 2+ | 2+ | − | − | − | − | + | − | 2+ |

2+, detection of a strong signal; +, detection of a weak signal; ±, detection of a very weak signal; −, no signal detected. The relative intensity of the immunoblotting signals was the average of several experiments.

*a*) E1A and E1B adenovirus-positive cells.

*b*) A deletion in exon 21 of the *RB* gene leads to an aberrant small-sized protein.

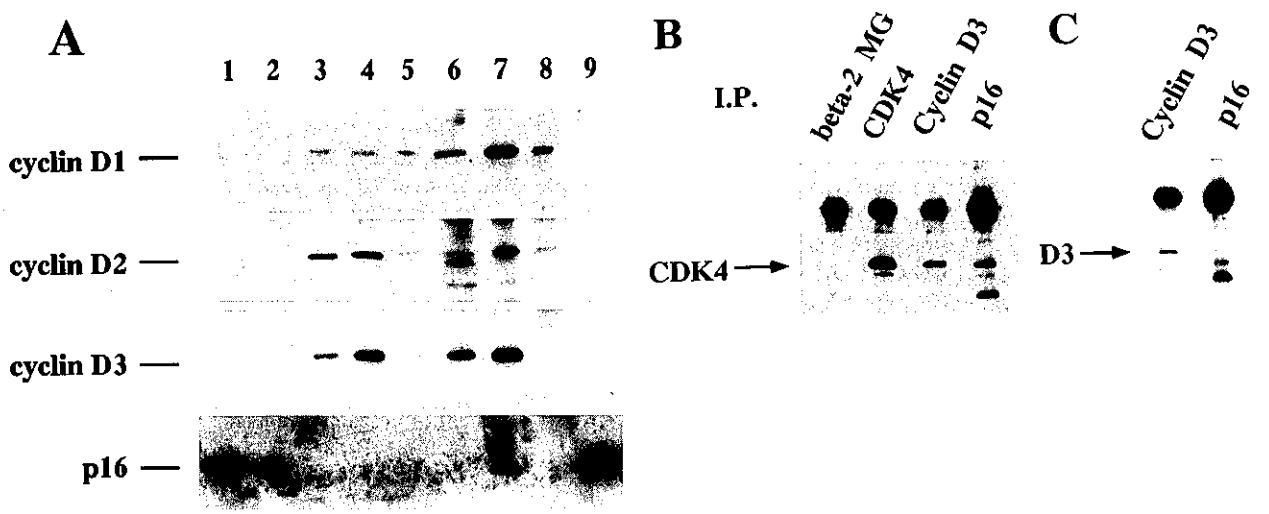


Fig. 2. The association of D-type cyclins, p16 and cyclin-dependent kinase (CDK)4 in urological tumor cell lines. A, Western blot analysis using anti-cyclin D1, D2, D3 and p16 antibodies of urological tumor cell lysates immunoprecipitated with anti-CDK4 antibody. Lane designations are as in Fig. 1. B, Western blot analysis with anti-CDK4 antibody of the immunoprecipitates with anti-$\beta_2$ microglobulin, CDK4, cyclin D3 and p16 antibodies of the cell lysate of LNCaP. Anti-$\beta_2$ microglobulin antibody was used as a negative control. C, Western blot analysis with anti-cyclin D3 antibody of the immunoprecipitates with anti-cyclin D3 and p16 antibody of the LNCaP cell lysate.

munoblotting, and they assembled with CDK4 independently. Stable coexistence of cyclin D/CDK4 and p16/CDK4 complexes was not expected because the half-life of cyclin D is very short.[7] It might be caused by moderate p16 expression induced by partial loss of repression of pRb. Hara *et al.* proposed that, in addition to relieving the repression exerted by pRb, the apparent overexpression of p16 in RB-negative cell lines was caused by another factor, an increase in the number of population doublings, judging from the high levels of p16 in senes-

cent cells.[20] However, our findings on LNCaP are incompatible to this hypothesis, because p16 expression in LNCaP was consistent regardless of the passage number.

The alteration of prostatic cancer to a hormone-refractory state is most serious in the clinical setting. LNCaP is the only human prostatic cancer cell line with wild-type p53 and pRb that remains androgen-responsive *in vitro* and produces glycoproteins such as prostatic-specific antigen as an indicator of differentiated character.[23-25] Therefore, LNCaP is a useful cell line for examining the mechanism of androgen-stimulation in prostatic cancer, and it has been studied in comparison with androgen-insensitive DU145 and PC-3 cell lines.[26, 27] Whether overexpression of p16 along with pRb plays a role for in the retention of androgen-sensitivity in prostatic cancer remains to be elucidated.

LNCaP has a slow growth rate.[23, 27] Indeed, the population doubling times of LNCaP, DU145 and PC-3 under our conditions were 63 h, 22 h and 26 h, respectively (unpublished results). The overexpression of p16 in the LNCaP cell line, that retains pRb expression, may brake the cell cycle progression and slow down the growth rate. It would be of interest to clarify the changes in cell cycle regulation after androgen-stimulation.

In conclusion, we observed the concomitant presence of p16/CDK4 and cyclin D/CDK4 complexes in the LNCaP prostatic cancer cell line. This suggests that partial pRb inactivation in LNCaP induces moderate expression of p16 that can partially interfere with the formation of cyclin D/CDK4 complex. The LNCaP cell line should be valuable as a model to study the dysregulation of the restriction point control in cancer cells.

We thank Dr. R. Takahashi for advice on immunoblotting of pRb and Drs. H. Yamada, M. Mishina and K. Mitsumori for kind discussions and help. We also thank Dr. S. I. Nishikawa for advice. This study was supported in part by grants from the Japanese Ministry of Education, Science, Sports and Culture and a grant from the Japan Society for the Promotion of Science.

*(Received October 29, 1996/Accepted January 22, 1997)*

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