

# Alteration of Cell Cycle in Cervical Tumor Associated with Human Papillomavirus - Cyclin-Dependent Kinase Inhibitors -

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The ability of viral oncoproteins to subvert cell cycle checkpoints may constitute a mechanism by which viral oncoproteins induce genetic instability. HPV 16 E6 and E7 disrupt cell cycle checkpoints, particularly affecting nearly all cyclin-dependent kinase inhibitors linked to the G1- and G2-checkpoints, in each case by means of a different mechanism. HPV 16 E7 shows homology with the pRb binding sites of cyclin D1, which consequently releases E2F. In addition, E7 directly binds to p21, and releases PCNA and other S-phase promoting genes. In turn, released E2F activates cyclin E, and cyclin E accelerates p27 proteolysis as a function of the antagonistic reaction of its own inhibitor. The induction of p16 expression is assumed to be indirectly associated with E7, which is upregulated only after prolonged inactivation of Rb.

HPV 16 E6 decreased the fidelity of multiple checkpoints controlling both entry into and exit from mitosis, with the mechanism of p53 inactivation. In addition, HPV 16 E6 increased the sensitivity to chemically induced S-phase premature mitosis and decreased mitotic spindle assembly checkpoint function.

Alongside the impressive advances made in the understanding of the molecular mechanisms, which HPV disrupts, the validity of these conclusions should be evaluated in the diagnostic and prognostic fields.

**Key Words:** Cyclin dependent kinase inhibitor, cervix cancer, human papillomavirus

## INTRODUCTION

The cyclin-dependent kinase (CDK) inhibitors

are divided into two groups. The INK4 family inhibits the cyclin/cdk complexes involving cdk4, cdk6, and cyclin D., expressed at the mid-G1 phase. Within this group, which includes p16, p15, p18 and p19, the first two CDK Inhibitors have been shown to have mutations and deletions in several kinds of tumors.<sup>1,2</sup> The second group of CDK inhibitors, the CIP/LIP family, includes p21, p27, and p57. They inhibit the cyclin/cdk2,cdk4 complexes and cause G1 arrest.<sup>3-5</sup> These proteins are rarely mutated in human tumors. Initially, p21 was thought to be induced by p53 only, but there is evidence that p21 can be regulated by p53-independent mechanisms.<sup>6</sup> Another important factor influencing the levels of CDK inhibitors is degradation of the proteins.<sup>4,7</sup> P21 and p27 have been extensively studied, and the determination of their expression levels is of prognostic significance in a wide spectrum of epithelial neoplasms of the gastrointestinal tract, breast, lung, prostate, urinary bladder, ovary, but less commonly in the case of cervical tumors.

Cervical cancer, caused by human papillomavirus (HPV) altering the cell cycle function, has its own well-known mechanism. The ability of high risk E6 and E7 to bind to and promote the degradation of the tumor suppressor protein p53, and to inactivate the hypophosphorylated, growth-suppressive form of the retinoblastoma tumor suppressor protein, pRb, respectively, are thought to play a critical function in promoting cellular transformation.<sup>8,9</sup> Unlike the disruption of G1 checkpoint caused by p53 inactivation, the ability of HPV oncoproteins to interfere with mitotic checkpoints has been less well studied, in terms of its possible

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association with p53 inactivation. Nonetheless, a number of studies suggest p53 is able to regulate the G2/M transition.<sup>10,11</sup> The G2/M transition is regulated by a different mechanism from that of the G1-S transition, the latter being linked to CDK inhibitors. Three gene functions have been shown to regulate p34<sup>cdc2</sup> activity at the initiation of mitosis, a negative regulator of *wee1* and *nim1*, with a positive regulator, *cdc25*. G2 checkpoint alteration in cervical neoplasms, specifically associated with HPV, needs to be elucidated.

In this article, we review and present current research on the CDK inhibitors associated with the G1- and G2-checkpoints in cervical neoplasms associated with HPV.

### Cyclin-dependent kinase inhibitors linked to G1 checkpoint

Cellular cyclin-dependent protein kinase (CDK) levels tend to remain in a state of constant excess throughout the normal cell cycle, and the regulation of catalytic activity is primarily post-translational.<sup>12</sup>

P21<sup>CIP1/WAF1/SDI1</sup> is a transcriptional target of p53 and is a critical determinant of the G1 arrest occurring in response to DNA damage.<sup>13-15</sup> However, p21 also responds to other signals independently of p53, and has been implicated in terminal differentiation and senescence.<sup>6</sup> This CDK inhibitor is unusual in that the two critical binding sites are mutually competitive, with the amino-terminal cyclin and CDK binding RRLFG motif, and the proliferating cell nuclear antigen (PCNA) binding RRLIF motif on its carboxy terminus.<sup>16</sup> HPV-16 E7 has the capacity of blocking the ability of p21 to inhibit CDK activity, as well as PCNA-dependent

DNA replication, through direct binding to the carboxy-terminus of p21, which is required for HPV DNA replication by promoting the G1-to-S phase transition.<sup>16</sup> The interaction with the carboxy-terminus of p21 modulates both its CDK- and PCNA-inhibitory function.

The main regulation of the intracellular levels of protein p16<sup>INK4a</sup> occurs at the transcriptional level.<sup>17</sup> The p16<sup>INK4a</sup> mRNA is extremely stable ( $T_{1/2} > 24$  hr),<sup>18</sup> and the half-life of the protein ranges from 8 to 18 hr, much longer than that of cyclin D1 (12 min).<sup>19</sup> Accumulation of p16<sup>INK4a</sup> mRNA and protein has been reported in response to inactivation of the retinoblastoma gene (*Rb*),<sup>20,21</sup> especially in the case of cervical carcinoma with HPV infection.<sup>22</sup> However, the link between p16<sup>INK4a</sup> and *Rb* is more complicated than was initially thought, because p16 expression does not simply reflect the status of *Rb*. The expression of p16 remains constant during the transition from quiescence to proliferation, and during progression through the cell cycle.<sup>23</sup> It is possible that the induction of p16 expression occurs only after prolonged proliferation in the absence of functional *Rb*. As in the case of *Rb*, the inactivation of p53 can also result in upregulation of p16 expression.<sup>17</sup> HPV 16 E6-containing fibroblasts continue proliferating despite the induction of p16, caused by inactivation of *Rb*, suggesting either that these cells have become independent of the *Rb* checkpoint or that the levels of p16 are not high enough to completely eliminate the CDK 4/cyclin D activity.<sup>17</sup> Our data regarding p21 and p16 in cervical tumors is shown in Table 1.<sup>24</sup> In the case of HPV infection, p16 was upregulated whereas p21 was downregulated in protein level.

P27 was initially discovered in cells arrested by transforming growth factor- $\beta$ , by contact inhibi-

**Table 1.** Expression of p21<sup>WAF1/CIP1</sup> and p16 in Patients with Cervical Carcinoma according to Lesion Size and HPV 16/18 Positivity

	No. of Cases	P21 index (%) (mean $\pm$ SE)	P16 index (%) (mean $\pm$ SE)
Lesion size			
<4 cm	12	27.4 $\pm$ 7.4	38.4 $\pm$ 6.6
$\geq$ 4 cm	34	24.6 $\pm$ 8.5	22.1 $\pm$ 9.2
HPV 16/18			
Negative	15	49.9 $\pm$ 8.7	12.2 $\pm$ 5.4
Positive	31	40.7 $\pm$ 6.3	35.2 $\pm$ 8.0*

\* $p < 0.05$ .

tion, and by lovostatin.<sup>25</sup> P27 acts in G0 and early G1 to inhibit G1 cyclin cdks, with the primary target being cyclin E/cdk2.<sup>26,27</sup> Once cyclin E-cdk2 is activated, it phosphorylates p27 to a form that is recognized by ubiquitin ligases and is targeted for destruction in proteasomes.<sup>1</sup> Therefore, cyclin E-cdk2 antagonizes the action of its own inhibitors.<sup>1</sup> P27 mRNA level is constant throughout the cell cycle and its protein level is regulated by translational controls and by ubiquitin-mediated proteolysis.<sup>28</sup> P27 may be inactivated upon binding to DNA tumor virus oncoprotein, such as HPV 16 E7.<sup>29</sup> P27 was found to be significantly lower in patients with HPV compared to those without HPV, as shown in Table 2 ( $p < 0.05$ ).<sup>30</sup> By contrast, the cyclin E index revealed increased expression in patients with HPV infection and was statistically significant in positive cases of HPV, compared to patients without HPV infection ( $p < 0.05$ ).<sup>30,31</sup> The expression of p27 was significantly lower in CIN when compared with normal tissue (p27 index: 65% vs 32% vs 24.8%, normal vs CIN vs carcinoma, respectively).<sup>30</sup> P27 can be deregulated early in carcinogenesis and may precede tumor invasion.<sup>27</sup>

### Cyclin-dependent Kinase Inhibitors linked to G2 checkpoint

Virally transformed cells are genomically unstable, exhibiting aneuploidy and chromosomal

rearrangements.<sup>32</sup> These oncoprotein expressing cells have clearly lost control of genomic integrity prior to immortalization. HPV-16 E6 and E7 have the ability to disrupt mitotic checkpoints in normal diploid human cells. Especially, E6 increased cdc2-associated histone H1 kinase activity, and increased sensitivity to S-phase premature mitosis and decreased mitotic spindle assembly checkpoint function relative to a control population.<sup>8</sup> The mechanism of disregulation of G2/M control, which is caused by HPV E6, influences kinase activity at a post-translational step distinct from cdk-cyclin complex formation.<sup>8</sup> HPV 16 E6 alters mitotic checkpoint fidelity through its effect not only on cdc2 activity, but also on reduced p21.<sup>22</sup> Alteration of the G2 checkpoint could be reflected by high immunoreactivity to a key regulator, cyclin B1/cdc2 complex, because of transcriptional upregulation and their stability.<sup>33</sup> The expression of cyclin B1/cdc2 complex was found to be significantly higher both in HPV-positive cases than HPV-negative cases ( $p = 0.000$ ), and in SCC than CIN ( $p = 0.000$ ) in Table 3.<sup>34</sup>

Whereas both antibodies were restricted to the suprabasal layer of the normal squamous epithelium, they were found to be spreaded to the entire epithelium in CIN, and along the frontal invading margin in SCC and in adenocarcinoma (Fig. 1).<sup>34</sup> When this complex is inappropriately abundant in tumor cells, these cells fail to operate M-phase termination, which represents G2 delay.

**Table 2.** Expression of Cyclin E and p27<sup>KIP1</sup> in Patients with Cervical Carcinoma according to Lesion Size, Cell Type and HPV 16/18 Positivity

	No. of cases	CEI (%) <sup>a</sup>	P27 index (%) <sup>a</sup>
Lesion size			
< 4 cm	24	2.7 ± 3.4	38.4 ± 6.6
≥ 4 cm	21	7.2 ± 3.4	27.2 ± 3.6
Cell type			
Squamous	39	15.5 ± 5.2	45.3 ± 5.8
Adenocarcinoma	4	4.6 ± 5.9	50.1 ± 7.2
Adenosquamous	2	9.2 ± 12.5	39.1 ± 8.6
HPV 16/18			
Negative	14	19.9 ± 8.7	32.2 ± 5.4
Positive	31	40.7 ± 6.3 <sup>b</sup>	15.2 ± 8.0 <sup>b</sup>

CEI; cyclin E index.

<sup>a</sup>Mean ± SE.

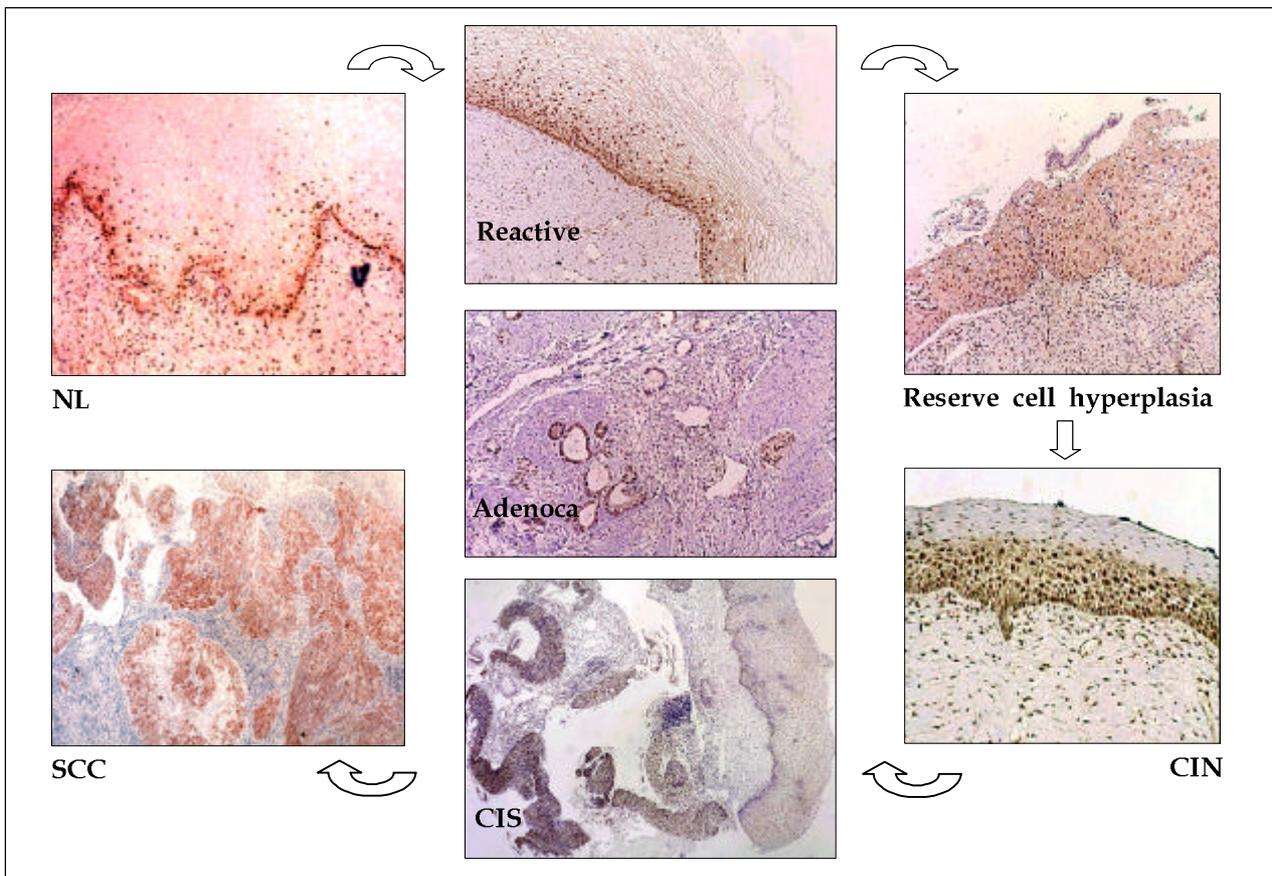
<sup>b</sup> $p < 0.05$  (HPV positive vs. Negative).

**Table 3.** Cyclin B/ p34<sup>Cdc2</sup> Expression in CIN and SCC according to HPV and between CIN and SCC

	Cyclin B		p34 <sup>Cdc2</sup>	
	Nc	Cyto	Nc	Cyto
HPV 16 (+)	26.4 ± 3.9*	14.7 ± 2.5*	18.9 ± 2.9*	35.2 ± 3.9*
HPV 16 (-)	8.2 ± 1.2	7.0 ± 1.0	5.6 ± 0.9	9.6 ± 1.3
CIN (n=102)	14.8 ± 2.0	11.5 ± 1.6	9.8 ± 1.4	17.9 ± 2.1
SCC (n=46)	33.3 ± 4.1*	21.8 ± 2.8*	24.6 ± 3.3*	42.9 ± 3.7*

NC, nucleus; Cyto, cytoplasm; CIN, cervical intraepithelial neoplasm; SCC, squamous cell carcinoma.

\*means statistically significant difference by less than 0.05 between two groups.



**Fig. 1.** The immunoreactivities of cyclin B1 and P34<sup>Cdc2</sup> in cervical neoplasm. They are proportionately increased as the disease progresses.

P34<sup>Cdc2</sup> acts as a universal regulator of the M phase in eukaryotic cells. Its regulatory genes<sup>31</sup> are *wee1*, *nim1*, *cdc25*, *p13<sup>sup1</sup>*, and *p56<sup>cdc13</sup>*.

Phosphorylation of T(Threonine) 14 and Y (Tyrosine) 15 is particularly important in the control of *cdc2* activation at mitosis, and roughly parallels the rise in cyclin B levels. Cdc2-cyclin B

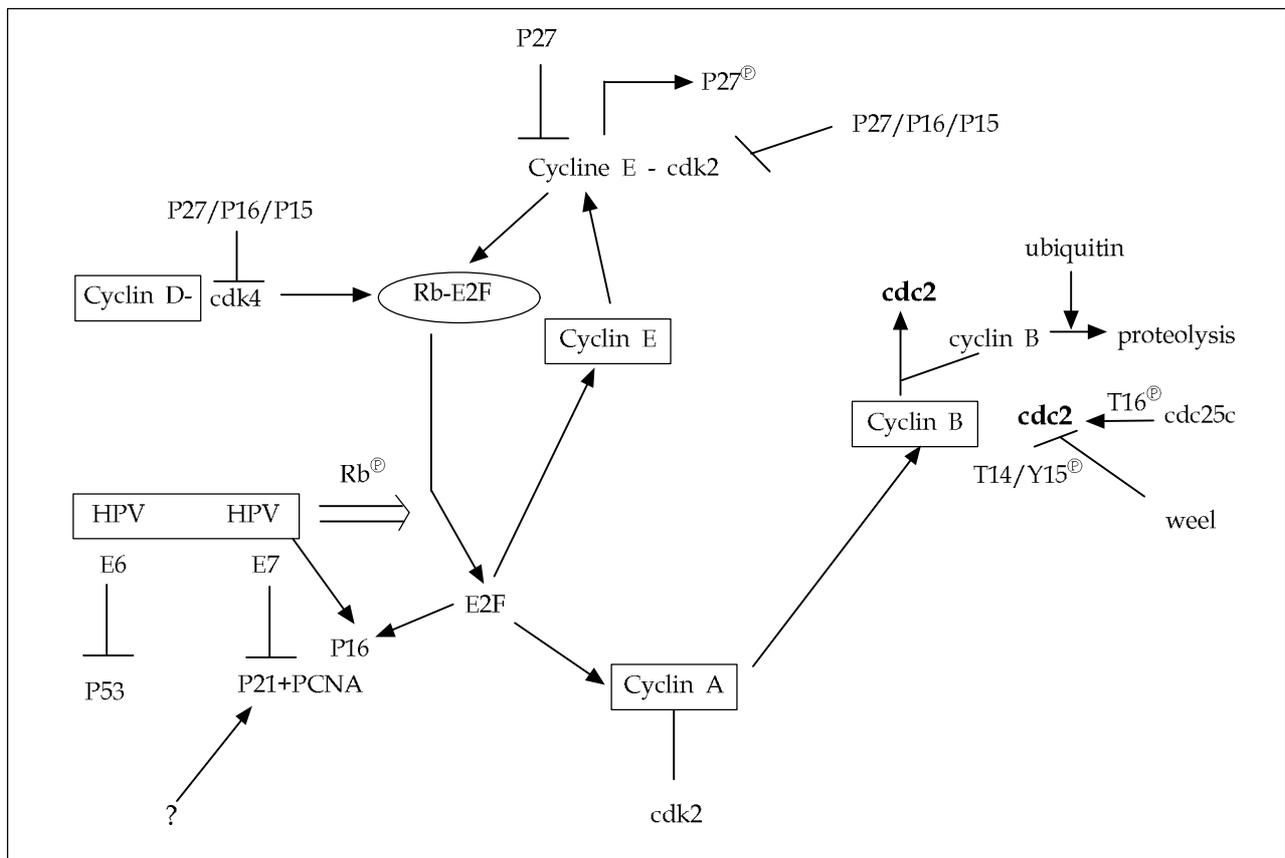
complexes are maintained in an inactive state, until T14-Y15 dephosphorylation by *cdc25* at the end of G2 activates *cdc2*.<sup>12</sup> In contrast to *cdc25*, the major candidate for Y15 kinase is *wee1*, whereas T14 kinase has not yet been elucidated.

P80<sup>Cdc25</sup> counteracts an inhibitory pathway and brings about activation of p34 through the dep-

phosphorylation of T14 and Y15, which is speculated to be dual-specific phosphatase.<sup>12</sup> However, this process cannot occur unless DNA replication is completed. Activation does not involve changes in *cdc2* transcript or p34 protein levels. The Cdc25-stimulatory kinase may be *cdc2* itself, forming the basis of an elegant positive-feedback system that could in theory induce the abrupt mitotic dephosphorylation of *cdc2*. Positive feedback may also be achieved by coordinated effects

on other components: *cdc2* may stimulate kinase that inactivates *wee1* and inhibit the phosphatase that inactivates *cdc25* and activates *wee1*.<sup>12,35</sup>

The inter-relationship among CDK inhibitors is summarized in Fig. 2. The cyclin D-cdk4 initiates Rb phosphorylation, releasing E2F, the products of which are necessary for S-phase entry. Activation of cyclin E by E2F enables the formation of the cyclin E-cdk2 complex, which is accelerated by the continued sequestration of p27 into complexes



**Fig. 2.** Restriction point control and G1-S/G2-M transition. As cells enter the division cycle from quiescence, the assembly of cyclin D-cdk4 complex in response to mitogenic signals requires p27 proteins, which are incorporated into catalytically active holoenzyme complexes. The cyclin D-cdk4 initiates Rb phosphorylation, releasing E2F from negative constraints and facilitating the activation of a series of E2F-response genes, the products of which are necessary for S-phase entry. Activation of cyclin E by E2F enables formation of the cyclin E-cdk2 complex. This is accelerated by the continued sequestration of p27 into complexes with the assembling cyclin D-cdk4 complex. Cyclin E-cdk2 complex completes the phosphorylation of Rb, further enabling activation of E2F-responsive genes, including cyclin A. Cyclin E-cdk2 also phosphorylates p27, targeting it for ubiquitination and proteosomal degradation. The initiation of the self-reinforcing E2F transcriptional program together with the degradation of p27 alleviates mitogen dependency at the restriction point and correlates with the commitment of cells to enter the S phase. In the subsequent cycle, the formation of cyclin B-cdk2 complex is symbolized as the entry of the mitosis. This complex accumulates in an inactive state during the S- and G2 phases. The kinase is kept inactive by phosphorylation of *cdc2* on Tyr-15 and Thr-14 by Wee1. At the end of G2, the *cdc25C* phosphatase is stimulated to dephosphorylate T14/Y15 and activates *cdc2* as part of a positive feedback loop. A number of ubiquitin-dependent proteolysis events are required for the cell to progress past anaphase, including the destruction of the B-type cyclins. In the case of HPV infection, E7 disrupts cell cycle not only by upregulation of p16, but also by downregulation of p21 and subsequent releasing of PCNA through direct binding to the C-terminus of p21. The fact that E7 releases E2F by hyperphosphorylation of Rb and E6 inactivates p53 are already proven both *in vitro* and *in vivo*.

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### Conclusion

HPV affects the cell cycle in several ways, wherein CDK inhibitors are altered. E7 inhibits p21 by directly binding to the specific region corresponding to the PCNA binding site in the carboxy terminus, where overlaps with the cyclin-cdk binding site, whereas E7 induces p16 expression indirectly, followed by the long-standing inactivation of hyperphosphorylated Rb. P27 expression is decreased when cyclin E is activated as a result of E2F release. On the other hand, E6 keeps p53 inactivated, which in turn results in G2 delay occurring, which is probably related to the cdc2 activation and cyclin B stabilization. The HPV oncoproteins decrease the vigilance of the mitotic checkpoint as well as that of the G1 checkpoint.

Given the complexity of the cell cycle machinery and its control mechanisms, the best approach to the further study would be to design a panel of markers that reflects the disruption of a certain pathway or control mechanism. With the information provided by the evaluation of these markers, pathologists should be able to complement morphologic diagnoses and thus help the clinician design more effective therapeutic regimens tailored to the molecular profile of a particular tumor.

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