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Aberrant Cell Cycle Regulation in Cervical Carcinoma

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Carcinoma of the uterine cervix is one of the most common malignancies among women worldwide. Human papillomaviruses (HPV) have been identified as the major etiological factor in cervical carcinogenesis. However, the time lag between HPV infection and the diagnosis of cancer indicates that multiple steps, as well as multiple factors, may be necessary for the development of cervical cancer. The development and progression of cervical carcinoma have been shown to be dependent on various genetic and epigenetic events, especially alterations in the cell cycle checkpoint machinery. In mammalian cells, control of the cell cycle is regulated by the activity of cyclin-dependent kinases (CDKs) and their essential activating coenzymes, the cyclins. Generally, CDKs, cyclins, and CDK inhibitors function within several pathways, including the $p16^{INK4A}$-cyclin D1-CDK4/6-pRb-E2F, $p21^{WAF1}$-$p27^{KIP1}$-cyclinE-CDK2, and $p14^{ARF}$-MDM2-p53 pathways. The results from several studies showed aberrant regulation of several cell cycle proteins, such as cyclin D, cyclin E, $p16^{INK4A}$, $p21^{WAF1}$, and $p27^{KIP1}$, as characteristic features of HPV-infected and HPV E6/E7 oncogene-expressing cervical carcinomas and their precursors. These data suggested further that interactions of viral proteins with host cellular proteins, particularly cell cycle proteins, are involved in the activation or repression of cell cycle progression in cervical carcinogenesis.

Key Words: Cell cycle, cervical carcinoma, cyclin

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INTRODUCTION

Carcinoma of the uterine cervix is one of the most common malignancies among women worldwide. The incidence of cervical carcinoma is on the decline, but still 370,000 new cases and 190,000 resulting deaths occur each year.[1] The development of cervical carcinoma is a multi-step carcinogenesis. However, the steps in the progression from low-grade cervical intraepithelial neoplasia to carcinoma remain unknown. Human papillomaviruses (HPVs) have been identified as the major etiological factor in cervical carcinogenesis.[2] Epidemiological evidence indicates that the majority of cervical neoplasia is attributable to HPV infection. Also, although certain HPV genes are capable of immortalization and can cooperate in the process of transformation, not all non-invasive lesions progress to the full malignant phenotype. This suggests that other cofactors are required.

Recently, it has become increasingly clear that HPVs replicate by interfering with normal cell cycle control mechanisms. Malignant transformation is also intimately related to these processes, and it is likely that the oncogenic potential of papillomaviruses lies in their ability to alter cell cycle checkpoints, thereby leading to the accumulation and transmission of genetic abnormalities. As we know, the E6 and E7 oncoproteins of high-risk HPVs, particularly HPV 16 and 18, bind respectively to the p53 and Retinoblastoma (Rb) tumor suppressor proteins, which are involved in the regulation of growth control. Moreover, the E6 proteins of high-risk HPVs bind more effectively to the p53 protein, leading to their degradation via a ubiquitin-mediated pathway, than those of lower-risk HPVs.

The development and progression of cervical carcinoma has been shown to be dependent on various cellular genetic and epigenetic events, especially alterations of the cell cycle machinery at various checkpoints. The precise control of the cell cycle in mammalian cells is regulated by the activity of cyclin-dependent kinases (CDK1, CDK2, CDK4, CDK6) and their essential activating coenzymes, the cyclins (cyclins A, B, D, E). The kinase activities of these CDKs are regulated by the abundance of their partner cyclins, phosphorylation by various kinases, de-phosphorylation by cell cycle phosphatases, and interaction with CDK-inhibitory proteins (CDKIs).[3-5]

The CDK family is an important group of molecules that regulate cell proliferation. In addition, two classes of mammalian cyclin-dependent kinase inhibitors (CDKIs) have been described: the CIP/KIP family, comprised of p21, p27, and p57, and the INK4 family, comprised of p15, p16, p18, and p19.[6] The INK4 molecules specifically inhibit cyclin D complexes by interaction with the CDK4 and CDK6 components. The KIP family is promiscuous, affecting cyclin E, cyclin A/CDK2, and cyclin B/CDK1 by binding both the cyclin and CDK subunit.[3]

In this article, we review the current knowledge of the role of cell-cycle regulatory proteins and their alterations in cervical cancer. CDKs, cyclins, and CDKIs generally function within several defined pathways, including the $p16^{INK4A}$-cyclin D1-CDK4/6-pRb-E2F pathway, the $p21^{WAF1}$-$p27^{KIP1}$-cyclinE-CDK2 pathway, and the $p14^{ARF}$-MDM2-p53 pathway.[7] Each of these components plays either a positive or a negative role in cell- cycle control mechanisms in cervical carcinogenesis. Alterations in CDKs, CDKIs, and cyclins can lead to uncontrolled proliferation and might contribute to malignant transformation of the uterine cervix.

The $p16^{INK4A}$ -cyclin D1-CDK4/6- pRb-E2F pathway

$p16^{INK4A}$

The $p16^{INK4A}$ gene maps to 9p21, contains three exons, and encodes a nuclear phosphoprotein with a molecular weight of 16 kDa. The p16 protein functions in the negative regulation of the cell cycle through the inhibition of cyclin- dependent kinases 4 and 6 and interactions with cyclin D1.[8] In the absence of p16, CDKs bind to cyclin D1, and the Retinoblastoma protein (pRb) is phosphorylated. Phosphorylation of pRb leads to its deregulation at the G1/S checkpoint, and cell proliferation is switched on. In a variety of human malignant tumors and cell lines, the $p16^{INK4A}$ gene is inactivated by various genetic mechanisms, including point mutations, homozygous deletions, and hypermethylation of CpG islands in the $p16^{INK4A}$ promoter. Kim *et al.* found a high percentage of p16 exon 2 mutations in cervical cancer specimens.[9] Dong *et al.* and Nuovo *et al.* also described hypermethylation of the p16 promoter, documenting inactivation of the gene as a frequent epigenetic event in cervical carcinoma.[10,11] However, mutations in p16 in these lesions have not been confirmed.[10] In some reports, mutation and deletion of p16 are suggested to be rare events: no gene alterations were detected in most studies of primary cervical carcinomas or cervical cancer cell lines, indicating that they are not required for the development of this tumor type. These data are concordant with those of previous reports.[12-14]

Conflicting results about the expression of p16 protein have been obtained from studies of the role of p16 in the pathogenesis of cervical cancers.[9,13,15,16] Overexpression of the p16 protein is a characteristic of displastic and neoplastic alterations of the cervical epithelium. The portion of p16-positive samples increases as the tumor progresses from the CIN I to the invasive carcinoma stage.[16] Some reports have shown that p16 expression is detectable by immunohistochemistry in cervical neoplasia and that this expression may be a direct result of HPV infection with resultant inactivation of pRb, which is known to bind p16.[15,16] Squamous cell carcinomas (SCCs), high-grade squamous intraepithelial lesions (HSILs), and adenocarcinomas (ACs) of the cervix have shown increased p16 immuno-staining.[17] Klaes et al. examined the utility of p16 immunostaining to assess inter-observer agreement in the diagnosis of cervical intraepithelial neoplasia (CIN). In that study, 194 cervical cone biopsies were examined and stained with an antibody to p16. A significant increase in inter-observer agreement when examining p16 expression was observed. The p16 expression was restricted to cervical cancer, CIN

2-3, and those cases of CIN 1 associated with high-risk HPV types.

Kelley *et al.*, however, reported that no alterations in p16 expression were detected in cervical cell lines, including 8 HPV-positive cell lines, 2 HPV-negative cell lines containing mutant Rb, and one tumorigenic cell line derived from normal cervical cells following transfection with HPV-16 and v-H-ras (CX16-2HR).[14] These results confirm that mutational inactivation of $p16^{INK4}$ is a rare event in tumor samples with compromised Rb activity. Volgareva *et al.* reported that all stages of CINs and carcinomas analyzed were heterogeneous with respect to p16 expression. Samples that showed expression of p16 in 25% of cells or more were identified in a side-by-side study with samples that stained poorly or lacked staining. These samples are evidence that p16-negative cervical neoplasms and carcinomas do exist.[18]

The molecular basis for overexpression of p16 in cervical squamous carcinoma remains unclear. Some explanations include a genetic mutation or a supragenetic event at the protein level, such as increased half-life of the p16 protein. The interaction of p16 with pRb is thought to be central to the role of p16 in controlling cell cycle progression. It has been suggested that HPV infection leads to HPV-E7 binding to pRb, which in turn results in increased p16 expression. Some studies have suggested that this process only occurs in cases of infection by high-risk types of HPV: p16 transcription may also be directly induced by the transcription factor E2F released from pRb after binding of the viral oncoprotein E7.[19]

p16 has been used as a biomarker for dysplasia in the diagnosis of cervical squamous lesions and has the potential to be used as an additional screening tool.[8,20,21] The overexpression of p16 is closely associated with high-risk HPV infection and high-grade CIN.[22] A recent report showed that in cervical biopsy specimens, the staining pattern of p16 and a high percentage of p16-positive cells are closely related to infection with high-risk HPV types 16 and 18, and with CIN 2/3. Several studies of p16 in liquid-based cytology specimens suggested that p16 could be used in cervical screening as a marker for persistent high-risk HPV infection, and HSIL could also be useful in resolving ambiguous cases involving a differential diagnosis of cervical neoplasia (Table 1).[23,24]

Cyclin D1

The cyclin D1 (PRAD-1, CCND-1) gene maps to 11q13 and shows the characteristics of a cellular oncogene. Expression of cyclin D1 moderately oscillates throughout the cell-cycle, reaching peak levels in G-phase.[7] Cyclin D1 serves as a key sensor and integrator of extracellular signals in early to mid-G1 phase, mediating its function through binding the CDKs, histone acetylase, and histone deacetylases to modulate local chromatin structure around the genes that are involved in regulation of cell proliferation and differentiation.[25] In addition to CDK-binding functions, a body of evidence now indicates that D-type cyclins have CDK-independent properties. These properties are important for cellular growth, metabolism, and cellular differentiation. Cyclin D1 forms physical associations with more than 30 transcription factors and transcriptional co-regulators.[25] Several nuclear receptors, including the androgen receptor (AR), estrogen receptor (ER), thyroid hormone receptor, and peroxisome proliferator-activated receptor (PPAR), bind directly to cyclin D1 within cultured cells.[26,27] Both basal and ligand-dependent transactivation of nuclear receptors is regulated by cyclin D1.

Genetic aberrations in the regulatory circuits that govern transit through the G1 phase of the cell cycle occur frequently in human cancer, and overexpression of cyclin D1 is one of the most commonly observed alterations.[25] One model suggests that the overexpression of cyclin D1 may serve as a driving force through its cell-cycle regulating function. Cyclin D1 is amplified and/or overexpressed in a substantial proportion of different human tumors.

The role of cyclin D1 in cervical carcinogenesis is not clearly understood, and controversial results have been described. Cho *et al.* found that cyclin D1 levels were significantly lower in HPV-positive HSIL, invasive SCC, or adenocarcinoma compared to HPV-negative cases and normal cervical epithelium, consistent with other authors' results.[28-30] Contrary to this, the results of Nichols *et al.* described elevated cyclin D1 mRNA levels in

Table 1. Expression Status of p16 in Squamous Cervical Carcinoma

Reference No.	Pathology	No. of cases	Expression status	Technique
8. Murphy et al.	cGIN	12	100% positive expression	Immuno-staining
	CA	11	100% positive expression	
9. Kim et al.	SCC	20	Mutations in exon2	PCR-SSCP
10. Dong et al.	SCC	31	39% methylation	MSP
	CA	22	18% methylation	
12. Tsuda et al.	CIN	42	14.3% positive expression	Immuno-staining
	IC	53	43.4% positive expression	
13. Hirama et al.	SCC	41	No deletions or mutations	Southern blot/SSCP
	CC cell line	8	No deletions or mutations	
15. Sano et al.	SCC/CIS	34	100% strong positive	Immuno-staining
	GCA	16	100% positive expression	
	DC	15	100% positive expression	
16. Klaes et al.	CINI	47	100% overexpressiom	Immuno-staining
		7 (LR-HPV)	No expression	
	CINII	32	100% overexpressiom	
	CINIII	60	100% overexpressiom	
	IC	60	97% overexpressiom	
18. Volgareva et al.	CINI	51	37.3% positive expression	Immuno-staining
	CINII	38	31.6% positive expression	
	CINIII	24	66.7% positive expression	
	IC	21	95.2% positive expression	
	AC	5	100% positive expression	
21. Murphy et al.	cGIN	5	100% positive expression	Immuno-staining
	CINI	33	100% positive expression	
	CINII	38	100% positive expression	
	CINIII	46	98% positive expression	
	IC	10	100% positive expression	
22. Guo et al.	LSIL	107	57.9% positive expression	Immuno-staining
	HSIL CIN2/3	103	97.1% positive expression	
23. Saqi et al.	LSIL	27	74% positive expression	Immuno-staining
	HSIL	10	90% positive expression	
	SCC	1	100% positive expression	
24. Hu et al.	CINI	45	44% positive expression	Immuno-staining
	CINII	46	93% positive expression	
	CINIII	51	100% positive expression	

cGIN, cervical glandular intraepithelial neoplasia; CA, Cervical adenocarcinoma; SCC, squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial; HSIL, high-grade squamous intraepithelial; IC, invasive carcinoma; CIS, carcinomas or carcinoma in situ; GCA, genital condyloma acuminate; DC, dysplasia of the cervix; LR-HPV, low-risk HPV; MSP, methylation-specific PCR; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.

invasive cervical cancer that were not associated with increased protein amounts. Additionally, underexpression of cyclin D1 was shown to be regulated at the transcriptional level in another

study.[29-32] Almost all research data suggests that cyclin D1 expression is not found to correlate significantly with clinicopathological factors (histological type, tumor grade and stage, patient's age).[33,34] Conversely, despite an underexpression of cyclin D1 in cervical carcinomas compared to normal cervical epithelium, positive immunostaining, which was found in 28-32% of invasive carcinoma samples, was significantly associated with poor disease-free and overall survival.[29,34] The latter report is the only one showing cyclin D1 gene amplification in cervical carcinomas (24%), but this was not correlated with overexpression.

Contradicting results have been reported about overexpression of cyclin D1 in immunostaining studies of cervical carcinoma. One study showed overexpression of cyclin D1 in 3% of patients, while underexpression was reported in another study.[31,33] No significant increase in cyclin D1 protein levels, despite overexpression of cyclin D1 mRNA demonstrated by *in situ* hybridization, was reported in cervical carcinoma.[28,29] These discrepancy might be attributed to the use of different antibodies, different scoring criteria for the detection assay, and the varying tumor tissue characteristics in different studies. In addition, some studies showed that the level of cyclin D1 was significantly lower in CIN and SCC compared with normal epithelium and that these levels correlated significantly with HPV positivity.[28,33] Also, there is a low prevalence of G1 cyclins in cell lines with a mutated *Rb* gene, and DNA tumor virus infection can supplant tumor cell requirements for cyclin D1 protein.

Cyclin D1 levels are reported as significantly lower in HPV-positive LSIL, HSIL, invasive SCC, or AC compared to HPV-negative cases and normal cervical epithelium.[28,30,35] Cyclin D1 and HPV E7 possess similar binding regions for pRb and pRb-related pocket proteins, and inactivation of pRb either by the cyclin/CDK complexes in G1 or by interaction with the high-risk HPV oncoprotein E7 may result in a decreased expression of cyclin D1 (Table 2).

CDK4

The D-type cyclins (D1, D2, and D3) and their catalytic partners CDK4 and CDK6 act early in the G1 phase of the cell cycle.[3] Mitogen-induced signal transduction pathways promote the activation of cyclin D/CDK complexes at many levels, including gene transcription, cyclin D translation and stability, assembly of D cyclins with their CDK partners, and import of the holoenzymes into the nucleus, where they ultimately phos-

Table 2. Expression Status of Cyclin D1 in Squamous Cervical Carcinoma

Reference No.	Pathology	No. of cases	Expression status	Technique
28. Cho et al.	CIN	22	CI 0.87% underexpression	Immuno-staining
	SCC	39	CI 5.88% underexpression	
29. Bae et al.	CINIII	31	3% underexpression	Immuno-staining
	SCC	32	28% underexpression	
30. Southern et al.	LSIL	26 (LR HPV)	92% overexpression	Immuno-staining
		29 (HR HPV)	87% overexpression	
31. Nichols et al.	LSIL	5	60% overexpression	Southern blot/SSCP
	HSIL	8	12.5% underexpression	
	SCC	18	77.8% overexpression	Immuno-staining
32. Kurzrock et al.	SCC cell line	10	No expression	Northern blot
33. Skomedal et al.	SCC	74	3% underexpression	Immuno-staining
34. Cheung et al.	SCC	60	32% overexpressiom	Immuno-staining
*Kim et al.	SCC	41	Not different	RT-PCR/ Western blot

CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma; LSIL, low-grade squamous intraepithelial; HSIL, high- grade squamous intraepithelial; LR HPV, low risk HPV; HR HPV, high risk HPV; CI, cyclin index; RT-PCR, real-time polymerase chain reaction. a not published. *Authors of this review.

phorylate their substrates. The cyclin D-dependent kinases (CDK4 and CDK6) can phosphorylate Rb family members (Rb, p107, and p130), thus helping to inactivate their transcriptional corepressor activities.

Aberrantly expressed CDK4 could play an important role in cervical tumorigenesis. It is postulated that CDK4 oscillates between the INK4 and KIP inhibitors, blocking their suppressor activity. In cervical cancer, the demonstrated lower levels of INK4 molecules and the high levels of CDK4 would favor binding of the more abundant KIP inhibitors to these kinases, undermining their inhibition of cyclin E. Thus, in this situation Cyclin D is expendable. The E7 would deregulate pRb initially, unleashing E2F-induced cyclin E expression; the overexpressed CDK4 would tether the KIP molecules, allowing cyclin E to become sufficiently active to phosphorylate and inactivate pRb and p27, perpetuating its own activity and that of E7.[4,36,37]

Yoshinouchi *et al.* found overexpression of CDK4 in 72.6% of cervical cancer specimens;[38] this value was consistent with previous studies of cervical carcinoma.[33,34] In another study, CDK4 gene amplification was described in 25% of cervical cancers, whereas no mutations in exon 2 of the CDK4 gene were found.[34] To determine whether alterations of p16 might be involved in HPV-positive cervical cancers, Yoshinouchi looked for gene alterations and changes in the ability of the p16 protein to interact with CDK4 in 5 cervical cancer cell lines. No alteration of this gene was detected, and the p16 and CDK4 proteins were normally expressed. Additionally, the ability of p16 to interact with CDK4 was not abrogated in these cell lines. These cell lines were HPV-positive and carried wild-type p53 genes. These findings suggest that phosphorylation of pRb by CDK4 is not critical in the carcinogenesis or in the establishment of HPV-positive cervical cancer cell lines, since the HPV viral-transforming proteins E6 or E7 inactivate p53 and pRb tumor suppressor protein function, resulting in deregulated progression of the cell cycle.[38] So far, research studies have not found a correlation between amplification and overexpression of CDK4 and patient age, histological tumor type, or tumor grade or stage.[33,34]

To release cells from G1 arrest and to promote entry into S-phase, pRb is phosphorylated, and thereby inactivated, by the cyclin D1/CDK4 complex. This inactivation may also be achieved by the interaction of pRb with the viral oncoprotein E7, leading to the hypothesis that up-regulation of positive regulators upstream of pRb might be dispensable in high-risk HPV-infected cervical carcinomas. Interestingly, very recent work has indicated that cyclin D/CDK4 complexes also phosphorylate Smad3, negatively regulating the functions of transcriptional complexes that mediate cell growth inhibition by proteins of the TGF family.[39] Importantly, several lines of evidence indicate that cyclin D/CDK complexes play a second noncatalytic role in G1 progression by sequestering proteins of the Cip/Kip family, including $p27^{KIP1}$ and $p21^{CIP1}$, two potent inhibitors of CDK2.[40,41]

pRb/E2F

The retinoblastoma tumor suppressor gene (Rb) encodes the nuclear phosphoprotein pRb (p105), which has been found mutated or deleted in several types of human cancer. p105Rb and other Rb family members p107 and p130 regulate the activity of E2F transcription factors.[42] Complexes consisting of E2F and hypophosphorylated p105Rb repress the transcription of genes that are required for cell cycle progression, and repression is relieved by CDK-mediated phosphorylation of p105Rb.[43] pRb is subject to regulation by many factors, including E2F and cyclin D1. The hypophosphorylated pRb, complexed with a transcription factor, serves as a transcriptional activator of cyclin D1 by binding to its promoter. On the other hand, inactivation of pRb by phosphorylation via the cyclin D/CDK complex in late G1 would not only unleash E2F transcription factors, but would also decrease cyclin D1 expression.[3,44] D-Type cyclins interact with pRb pockets through their NH2-terminal L-X-C-X-E motifs. These are similar to DNA virus oncoprotein region 2, such as region 2 from HPV E7.[45] Thus, the oncoprotein may occupy the pRb pocket and displace E2F factors, preventing pRb/E2F from inducing cyclin D1 transcription and undermining its normal growth-suppressive function.

Some immunohistochemical studies of pRb in

human cancer and in normal tissues, including the uterine cervix, have shown that pRb is expressed in mature and differentiated cells. Both binding and degradation of the Rb proteins by the HPV E7 protein are essential for sustained proliferation of HeLa cervical carcinoma cells, and E7 repression triggers senescence at least in part by activating the Rb pathway in both HeLa and HT-3 cells.[42] Salcedo *et al.* observed that the majority of cells in the proliferating basal layer of the normal epithelium displayed low levels of pRb. pRb immunostaining in invasive cervical lesions is frequently lower than in SIL.[43] This low expression of Rb may result from *Rb* gene mutations or downregulation mechanisms, but may also be related to pRb inactivation resulting from complex formation with high-risk HPV E7 oncoproteins.

Rb immunoreactivity was not affected by high-risk HPV infection in most studies of cervical squamous cell carcinoma.[46] In contrast, Rb staining was even more frequently found in SIL and invasive cancer (93%) compared to normal epithelium or reactive atypia (78%).[47] Mutations in the Rb gene seem to be rare events in cervical cancer.[46,48] In most studies, *Rb* gene expression did not strictly correlate with the HPV status,[49] although Scheffner *et al.* reported a higher *Rb* mutation rate in HPV-negative than in HPV-positive cervical cancer cell lines. These mutations resulted in aberrant proteins that were not phosphorylated and unable to complex with the adenovirus E1A oncoprotein.[50] Cyclin D1 is the regulatory subunit of the holoenzymes that phosphorylate and, together with sequential phosphorylation by cyclin E/CDK2, inactivate the cell-cycle inhibiting function of the pRb protein. The hypophosphorylated form of pRb, complexed with E2F, serves as an activator of cyclin D1 transcription by binding to its promoter, thus driving cyclin D1 in early and mid G1-phase of the cell cycle. As D-type cyclins and HPV E7 possess similar binding regions for pRb and pRb-related pocket proteins, inactivation of pRb either by the cyclin/ CDK complexes in G1 or by interaction with the high-risk HPV oncoprotein E7 may result in a decreased expression of cyclin D1 (Fig. 1).[43,51]

The $p21^{WAF1/CIP1}$-$p27^{KIP1}$-cyclin E-CDK2 pathway

$p21^{WAF1/CIP1}$

$p21^{WAF1/CIP1}$ is a cyclin-dependent kinase inhibitor that associates with a class of CDKs and inhibits their kinase activities, leading to cell cycle arrest and the dephosphorylation of pRb. A large body of evidence suggests that $p21^{WAF1/CIP1}$ plays an important role in cell fate decisions during growth and differentiation. The $p21^{WAF1/CIP1}$ protein is a p53-inducible protein that inactivates the cyclin/CDK complexes, blocking the cell cycle progression in the G1-S transition. $p21^{WAF1/CIP1}$ is expressed in cells undergoing either G1 arrest or apoptosis by p53-dependent or -independent mechanisms.[52,53] Mutations in the $p21^{WAF1/CIP1}$ gene were very rarely detected in cervical carci-

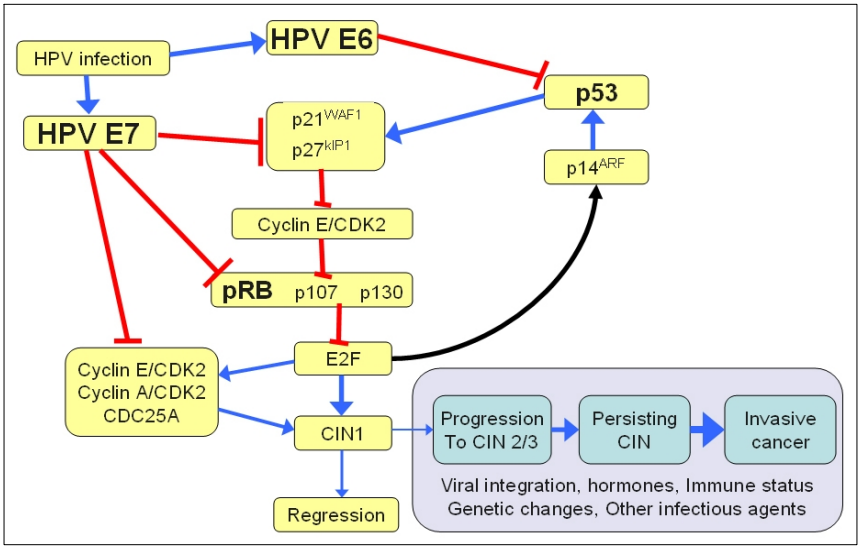

Fig. 1. Schematic presentation of the HPV viral oncoproteins E6/E7 and cell-cycle regulatory proteins in cervical carcinogenesis.

noma.

$p21^{WAF1/CIP1}$ expression usually correlates with favorable prognoses in ovarian, gastric, colorectal, and superficial bladder cancers and in esophageal squamous cell carcinoma. However, in cervical cancer, the conclusions about p21 expression and its prognostic importance vary considerably. Many authors found increased p21 expression in invasive carcinomas[33] and an increase in the number of p21-expressing cases during the progression from normal epithelia through precancerous lesions to invasive cervical cancer.[29,54] However, others detected an underexpression of p21 in micro-invasive and invasive cervical cancer compared to normal cervical epithelium.[55,56] Van de Putte *et al.*[57] did not find any expression of p21 in normal squamous cervical epithelium, in agreement with Giannoudis and Herrington[58] and Skomedal *et al.*,[33] but in contrast with other reports.[29,55] Longer antibody incubation and the use of chronic cervicitis as normal controls may partially account for the differences. However, increased expression of p21 in SCC compared to normal epithelium was found in all cases in the abovementioned studies.[29,33,55,59] An increase in p21 levels in invasive cancer seems at odds with its function of cell cycle control. However, the p21 level is probably increased in a futile attempt to overcome its impaired or bypassed function. In squamous cell carcinoma, its function could be impaired through the inactivation of p21 by the HPV-16 E7 oncoprotein. Other possibilities include inactivating mutations, mutation of its targets, overexpression of its targets sufficient to titrate p21, or overexpression of proteins in more downstream pathways in cell cycle control.[29]

On the other hand, Lu *et al.* reported that expression of $p21^{WAF1/CIP1}$ was correlated with a favorable prognosis in adenocarcinoma of the uterine cervix.[60] In most studies, however, there was no significant association of p21 expression and clinicopathological factors or prognosis.[29,55,61,62] Although the literature on the prognostic significance of p21 expression in other human cancer types is extensive, no clear picture can be drawn.

$p27^{Kip1}$

$p27^{Kip1}$ is a negative regulator of the G1 phase of the cell cycle. The $p27^{Kip1}$ gene is a tumor suppressor gene and is frequently lost in tumor cells. It has been implicated in the negative regulation of cell proliferation in response to extracellular signals and is induced upon serum deprivation. In normal epithelial cells, increased expression of $p27^{Kip1}$ mediates the arrest of cells in the G1 phase of the cell cycle when induced by TGF-β, contact inhibition, or growth in suspension.[63] p27 associates mainly with the cyclin E/CDK2 complex and, through this complex, inhibits pRb phosphorylation. Overexpression of p27 blocks the cell from entering S-phase.[64] Although $p27^{Kip1}$ is a putative tumor-suppressor gene, mutation or homozygous deletion of this gene is rarely found in human cancer.[65] Evidence that p27 may be involved in human tumor progression comes largely from studies that have directly measured the expression of p27 protein in clinical tumor samples using immunohistochemical assays. A decrease or absence of p27 protein expression has been shown to be associated with more aggressive clinical behavior in a variety of human tumors, including breast, lung, colon, gastric, and ovarian carcinoma.[65]

Goff *et al.* found that p27 was strongly expressed in the normal cervix, in both the epithelium and the stroma.[66] Other investigators have also found high levels of p27 expression in normal cervix.[33,67] Shiozawa *et al.* revealed that the expression of p27 in the normal squamous epithelium of the uterine cervix was observed mainly in the cells of the intermediate and superficial layers, but the expression of p27 was weak in the parabasal cells, which replicate most actively.[64] This is consistent with a previous study by Troncone *et al.*[68] In contrary, some studies have indicated that p27 expression is significantly lower in patients with cervical carcinoma compared with normal epithelium and dysplasia.[67,68] Huang *et al.* showed that expression of this protein was absent in 55.3% of the invasive carcinoma tumors.[65] The trend of reduced p27 expression in microinvasive and invasive carcinomas further supports the notion that p27 plays a tumor suppressor function during neoplastic transformation in cervical epithelium.

Sgambato demonstrated that a decrease in p27 expression is associated with the development of cervical cancer and may play an important role in the early stage of cervical tumorigenesis.[69] In fact,

in that study, p27 expression was already reduced in preinvasive lesions of the cervix and became progressively more evident during the progression from low- to high-grade SIL and from SIL to carcinoma. This result of p27 expression in cervical carcinomas is in agreement with similar findings in several other types of human malignancies[63] and with previous reports of cervical cancers.[64,65,68]

The lower levels of p27 in invasive cervical carcinomas compared with normal epithelium might be explained simply as a direct consequence of the increased cell proliferation, reflecting the fact that the time spent by the neoplastic population in the early part of the cell cycle is reduced.[68] Shiozawa *et al.* demonstrated that p27 seemed to be involved in the regulation of growth and differentiation in the normal squamous epithelia of the uterine cervix.[64] Thus, the apparently normal function of p27 may be preserved in preinvasive CIN lesions. However, invasive SCC samples showed a paradoxic overexpression of p27, which may represent aberrant regulation and/or function of the p27 protein.

Most studies indicate that low levels of p27 are not found to be predictive for reduced survival and do not correlate with age, menopausal status, tumor stage, grade of differentiation, histological type, or HPV infection.[33,62,65,67] However, Huang *et al.* demonstrated that loss of p27 expression was significantly associated with lymph node metastasis.[65] Contrary to that, in a study by Dellas *et al.*, high levels of p27 were associated with poor survival in invasive cervical carcinomas of clinical stage IB.[70]

In summary, $p27^{Kip1}$ expression is often shown to be detectable only in a small percentage of cervical cancers[33,67] and to be lower in cervical carcinoma compared with normal epithelium and precancerous lesions.[62,64,65,68] No relationship was observed between expression of $p27^{Kip1}$ and proliferative activity in cervical cancer as well as in other cancers, suggesting that deregulated expression of $p27^{Kip1}$ might contribute to tumor formation through mechanisms other than increased cell proliferation.[64,68]

Cyclin E

Cyclin E exists in two isoforms with high homology, designated cyclin E1 and E2. No major differences in expression or function between cyclin E1 and E2 have been found, and their expression has been assumed to be governed by the same molecular circuitry.[71,72] Cyclin E, whose catalytic partner is CDK2, is another rate-limiting regulator of the G1 phase of the cell cycle,[73] and increased expression of cyclin E has been found in several types of tumors. Appropriate regulation of this cyclin is essential for S-phase transition and numerous processes that determine the accuracy of chromosome replication. It can play a role similar to that of cyclin D1, driving the cell cycle by phosphorylation of Rb, p107, and p130 and the subsequent release of E2F and transcription of key proteins. Cyclin E appears in late G1 after passage through the restriction point. The level of cyclin E peaks in late G1 and disappears again in early S phase.

Even though gene amplification and post-transcriptional modification are the common causes of aberrant cyclin E expression in different malignancies, high-risk-HPV oncoprotein-associated mechanisms might contribute to the aberrant cyclin E expression in cervical lesions. Untethering of E2F factors by the action of the HPV E7 protein could result in the overexpression of cyclin E. This connection, however, is not enough to explain the highly expressed cyclin E in cervical epithelium. The HPV oncoprotein E7 was demonstrated in complexes with cyclin E and cyclin A, thus eliminating phase-dependent variation in activity. Also, the HPV E7 was found to interact with p21 and thereby block p21-mediated inhibition of cyclin E-associated kinase activity.[5,75]

In normal cervical squamous epithelium, cyclin E expression was not immunohistochemically detectable or was very weak.[28,64,67,74,76] However, cyclin E expression was increased in both low- and high-risk HPV-infected squamous and glandular lesions.[28,70] Cyclin E expression correlated strongly with morphologic features of the HPV-infected lesions, and has been observed in intermediate, and partly in superficial, cells from LSIL and HSIL, implying sequential dysregulation.[77] A positive correlation between cyclin E expression and cell proliferation rate in precancerous lesions was demonstrated by some studies.[70,78] In contrast, Kanai *et al.* showed lower proliferation rates

in cyclin E-positive than in cyclin E-negative squamous cervical carcinomas, and Quade *et al.* found cyclin E staining more frequently in more differentiated areas of invasive cancer.[74,79] HPV-infected cancer was found to express cyclin E significantly more frequently than HPV-negative cases, but this expression was not related to prognostically important factors including age, tumor size, and tumor type.[67] This is in accordance with the only other study on the prognostic significance of cyclin E in squamous carcinoma.

Cyclin E overexpression or over-activity may be the direct result of the presence of the HPV E6 and E7 proteins in invasive disease. The presence of HPV E7 leads to inactivation of pRb and increased transcription of cyclin E. The HPV E7 oncoprotein inactivates p27, leading to decreased inhibition of cyclin E.[75] Southern *et al.* reported an up-regulation of cyclin E and cyclin A expression in cases of both low-risk and high-risk HPV infection.[76]

CDK2

CDK2 probably functions as an effector through phosphorylation of key substrates such as the transcription factor p53, the cell cycle regulating phosphatases cdc25A/cdc25C, MDM2, BRCA1, and the transcription factor E2F1.[80] CDK2 can have an apoptosis-sensitizing effect and can arrest the cell cycle at different stages.

E-type cyclins (E1 and E2) govern the activity of the single catalytic subunit of CDK2. Unlike various combinations of D-type cyclins that are expressed in different cell types, cyclin E-CDK2 complexes are periodic and maximal at the G1- to S-phase transition.[81] Cyclin E-CDK2 also preferentially phosphorylates pRb at different sites from the cyclin D-dependent kinases, and these modifications may differentially impact on the interactions of pRb with E2Fs, histone deacetylases, and other chromatin-remodeling proteins.[82] One of the most significant consequences of pRb inactivation is activation of cyclin E/CDK2 subunits, often as a result of increased cyclin E expression. Cyclin E/CDK2 complexes can themselves participate in maintained inactivation of pRb in tumor cells.

The HPV oncoprotein E7 may abrogate p21-mediated sequestration of cyclin E and render its catalytic partner CDK2 resistant to p27.[83] Expression of CDK2 was observed in parabasal cells of normal squamous epithelium, which was inversely correlated to p27 expression. Shiozawa *et al.* revealed that no p27 expression was observed in atypical epithelial cells of SIL with elevated expression of Ki-67, cyclin E, and CDK2.[64] The expression of cyclin-dependent kinases (CDK2 and cdc2) was also increased in a considerable number of invasive cervical carcinomas.[64,79] In those cases, p27 expression was also high and retained the ability to bind to CDK2, but the p27/cyclin E/CDK2 complex still possessed phosphorylating activity.[64] CDK2 also regulates the cell cycle through its interactions with cyclin A2; thus its functions extend beyond G1 regulation to govern events in S and G2/M as well. Whereas maximal periodic cyclin E-CDK2 activity is detected at G1/S, low levels of cyclin A-CDK2 activity are first detected in late G1 phase.

Consistent with crucial roles for cyclin E/CDK2 downstream of pRb, many tumor cells are extremely sensitive to inactivation of cyclin E/CDK2 whether or not they express pRb. This conclusion has been drawn from a multitude of studies demonstrating antiproliferative effects of overexpression of $p27^{Kip1}$, a protein inhibitor of CDK2, or of dominant-negative CDK2 subunits. Further, injection of antibodies against the CDK2 activators cyclin E and cyclin A blocks proliferation, as does treatment of many different cells with CDK2 inhibitors.[84,85]

The $p14^{ARF}$-MDM2-p53 pathway

$p14^{ARF}$

The CDK^{N2A} gene on human chromosome 9p21 encodes two distinct proteins, $p14^{ARF}$ and $p16^{INK4A}$, which arise from the same gene by alternative mRNA splicing.[86] $p14^{ARF}$ has a mass of 15 kDa and is translated from mRNAs bearing a unique first exon, called 1-beta, located 15 kb upstream of the exon 1-alpha of $p16^{INK4A}$. Both transcripts share common exons 2 and 3. $p14^{ARF}$ functions as a cell cycle regulator, stopping cell growth at the G1-S border and also at G2-M.[87] $p14^{ARF}$ interaction inhibits the MDM2 oncoprotein, thereby blocking formation of the MDM2-p53 complex and preventing MDM2-induced p53 degradation.[88]

Kanao *et al.* reported that the overexpression of

$p14^{ARF}$ and $p16^{INK4A}$ is strongly associated with HPV-positive cervical cancers and that reduced expression of $p14^{ARF}$ and $p16^{INK4A}$ is correlated with HPV-negative cervical cancers.[86] These findings may indicate that impaired $p14^{ARF}$ and $p16^{INK4A}$ mRNA expression contribute to tumor development in HPV-negative cervical cancers by failure to support p53 and Rb instead of their inactivation by HPV E6 and E7.

In human fibroblasts, it has been shown that p14ARF-induced cell cycle arrest is p53- dependent and is abrogated by the co-expression of the HPV E6 protein.[89,90] Therefore, upregulation of p14ARF is a consequence of inactivation of p53 rather than a sign of cell cycle inhibition. Brooks *et al.* demonstrated that the expression levels of p14ARF were clearly elevated in 84% of cervical cancers and 79% of CIN III relative to matched normal tissue by RT-PCR.[91] Interestingly, immunocytochemical analysis revealed that $p14^{ARF}$ was abundantly expressed in almost all cases of both cervical SCC and CIN III and was overexpressed with p73 in a great majority of cases. The frequent simultaneous overexpression of $p14^{ARF}$ with p73 is consistent with E2F1-driven expression of both genes. p73 has structural and functional homologies to p53, including sequence-specific DNA binding and transactivation.[92]

MDM-2

The cellular protein MDM-2 is encoded by the *mdm*-2 oncogene and has been shown to bind to the p53 protein, inactivating its ability to function as a transcription factor.[93] MDM-2 is a zinc finger protein composed of 491 amino acids. The MDM-2 protein interaction with p53 to form the p53/MDM-2 complex covers the p53 N-terminal acidic or activation domain required for activation of transcription.[94] This is the same region of the p53 DNA binding domain bound by the HPV E6 protein, thus blocking transcriptional activation. Whether MDM2 acts solely through the inhibition of p53 or has additional activities remains to be determined.

The *mdm*-2 gene can be de-regulated in soft-tissue sarcomas, bladder carcinomas, renal-cell carcinomas, and breast carcinomas.[95-97] Dellas *et al.* analyzed the altered patterns of MDM-2 and p53 expression in cervical neoplasms and detected MDM-2 as well as p53 in all histological grades of cervical neoplasia.[95] The fraction of positive cells for MDM-2 ranged from 0 to 31% in CIN cases and from 0 to 81% in invasive carcinomas. The percentage of p53-stained neoplastic cells ranged from 0 to 40% in CIN cases and from 0 to 69% in invasive carcinomas. Dellas *et al.* demonstrated that aberrant MDM-2 and p53 expression is frequently found in cervical neoplasia. Skomedal *et al.* found that MDM2 may protect against HPV-induced p53 protein degradation.[95]

In contrast to the above-mentioned results, Lie *et al.* reported that there is no correlation between HPV status and expression of the cell cycle regulators p53, MDM-2, and p21.[54] Inactivation of p21 and p53 protein may be important, and MDM-2 abnormalities seem to play a minor role in the development of high-grade CIN. Ikenberg *et al.* found that p53 mutation and amplification of the *mdm*-2 oncogene are rare, even in HPV-negative primary cervical carcinomas.[98] In contrary, Tsuda *et al.* found strong expression of MDM-2 was higher in invasive cancers (32.1%) than in CINs (7.1%).[12] Troncone *et al.* reported that immunostaining for MDM-2 and $p21^{WAF1}$ was not abrogated in invasive cervical cancer by high-risk HPV genomic sequences, and there were no significant differences in the expression of p53, MDM-2, and $p21^{WAF1}$ between the HPV DNA-positive and -negative groups, as similar levels of expression were observed.[99]

p53

The *TP53* gene, present on chromosome 17p, acts as a tumor suppressor gene, controlling entry into the S-phase of the cell cycle, and p53 plays many important roles in cell proliferation. Mutation of this gene inactivates its suppressor activity and is related to tumor progression.[100] The p53 tumor suppressor gene encodes a transcriptional factor central in the regulation of cell growth, DNA repair, and apoptosis induction. Its activity requires the induction of several target genes, including MDM-2 and $p21^{WAF1}$.[99]

The *TP53* gene is lost or mutated in over 50% of human cancers. However, in contrast to many other human tumor forms, *TP53* mutations are only rarely detected in cervical cancer.[101,102] A great amount of research data suggests that the

inactivation of p53 is believed to play a major role in the carcinogenesis of the uterine cervix. Two different mechanisms may explain the loss of p53 function in cervical cancer-a somatic gene mutation which leads to an inactive form and/or the enhanced protein degradation promoted by the E6 oncoprotein of HPV type 16 and type 18. Horner *et al.* found that sustained inactivation of the p53 pathway by the E6 protein is required for maintenance of the proliferative phenotype of HeLa cervical carcinoma cells.[103] Troncone's results are consistent with several other reports, indicating that p53 immunostaining occurs in the vast majority of cervical cancers. This report also confirms that higher p53 protein levels are not dependent on the absence of high-risk viral infection as shown by the similar immunoreactivity scores of the HPV-positive and HPV-negative groups.[99,104,105] Tsuda *et al.* reported that the frequency of p53 overexpression was 28.6% of 42 CINs and 28.3% of 53 invasive carcinomas.[12]

On the other hand, some studies indicate that immunohistochemical p53 overexpression is not associated with survival in cervical carcinoma.[12,106] Other studies also show that there is no significant relationship between p53 expression and prognosis in squamous cell carcinomas of the uterine cervix. This is explained partly by the presence of HPV. The oncogenic potential of HPV appears to be mediated by the E6 and E7 proteins, which are known to bind and inactivate the p53 and pRb proteins, respectively. Furthermore, the HPV E7 oncoprotein has been shown to abrogate p21 and p27 function.[103,106,107] In addition to inactivation of the p53 and pRb proteins, other cell cycle regulatory proteins may be involved in the carcinogenesis of cervical cancer.

Other factors

Cyclin A

Cyclin A appears in the nucleus precisely at the G1/S transition, accumulates throughout the rest of interphase, and disappears at the beginning of mitosis. Both cyclin E and cyclin A control the progression through the cell cycle primarily by activating CDK2. Cyclin A is important for the G1-S transition, for progression through the S phase and for the G2-M transition. Cyclin A binds to CDK2 in S phase and to CDK1 (cdc2) during the G2-M transition. This complex induces phosphorylation of pRb and the nuclear membrane protein laminin, augmenting nuclear membrane disruption. Therefore, cyclin A is essential in the G1-S and G2-M phase transitions.[108] Forced expression of cyclin A provides anchorage independence to normal cells, and overexpression of cyclin A is a prognostic factor in different tumor types. The transcription of cyclin A is induced by E2F when it is untethered from pRb by the action of HPV E7 oncoprotein.[75] Cyclin A is overexpressed in cervical intraepithelial neoplasia and SCC compared to normal epithelium.[76,79] Van de Putte *et al.* found overexpression of cyclin A in SCC, consistent with previously described results.[57] Zehbe *et al.* demonstrated an overriding of cyclin-dependent kinase inhibitors by high- and low-risk HPV: cyclin A and $p21^{WAF1/CIP1}$ were co-expressed in the same cells, mainly in cases of low grade cervical intra-epithelial neoplasia (LCIN).[109] This provides strong evidence that the $p21^{WAF1/CIP1}$ G1 block can be overcome in HPV-infected cells *in vivo*. This conclusion is further strengthened by the result that cyclin A was never found during the G1 phase of cervical carcinoma-derived cell lines.

Cyclin B

During the normal cell cycle, cyclin B forms complexes solely with CDK1 (cdc2 or p34cdc2), forming the mitosis-promoting factor, which regulates the transition from G2 to M phase.[3,110] The mitosis-promoting factor is later translocated from the cytoplasm into the nucleus in late S phase. Recent evidence has shown that p53 regulates a G2 checkpoint through cyclin B: p53 prevents the G2/M transition by decreasing cyclin B protein levels and attenuating the activity of its promoter.[107]

Cho *et al.* found that cyclin B/p34cdc2 is significantly increased in HPV-16 or -18 positive cervical lesions, including CIN and SCC, in comparison with HPV-16 or -18 negative lesions.[107] Southern *et al.* demonstrated that expression of the cyclin B protein was up-regulated and persisted into the upper epithelial layers, in parallel with cyclin A expression, in HSIL infected with human papillomaviruses 16, 31, 33, 51, 58, 66, and 67.[77]

Kanai *et al.* also found elevated cyclin B levels in SIL and invasive carcinomas and concomitantly elevated levels of cdc2, indicating that cyclin B plays an important role in cervical carcinogenesis.[79] Contrary to the above results, Hashiguchi, *et al.* examined HPV status and cyclin B1 in CIN and invasive cancer by immunohistochemistry. This study showed that expression of cyclin B1 was 0% in CIN and 12.2% in invasive cancers, and the relationship between HPV type and cyclin B1 expression was not significant.[111] Further experimental data are needed to confirm the role of cyclin B in malignant transformation of the cervix.

CONCLUSION

Cervical cancer, which plagues women all over the world, is still a major focus of oncological research. Infection with HPV has been implicated as an important etiological factor in the development of uterine cervical cancer. Moreover, even in cases where HPV infection persists, cervical cancer most often does not appear until many years or more than a decade after infection. This long delay has supported the view that HPV infection may initiate, but not necessarily cause, progression to cervical carcinoma, and that oncogenic influences by other factors such as environmental factors, specific aspects of lifestyle, or genetic alterations are necessary to fully establish an HPV-induced malignancy. Because cancer is a multi-etiological disease, the time lag between HPV infection and the diagnosis of cancer also indicates that multiple steps as well as multiple factors may be necessary for the development of cervical cancer.

High-risk HPV types, specifically types 16 and 18, have been demonstrated to participate in cervical carcinogenesis through the expression of proteins, such as E6 and E7, interaction with different steroid hormones, and interaction with innate genetic susceptibilities to develop cervical cancer. The results from many studies showed aberrant alterations in the regulation of several cell cycle proteins, including cyclin D and E and p16, p21, and p27, to be characteristic features of HPV-infected and HPV E6/E7 oncogene-expressing cervical carcinomas and their precursors. These data suggest further that interactions of viral proteins with host cellular proteins, particularly cell cycle proteins, are involved in activation or repression of cell cycle progression in cervical carcinogenesis.

REFERENCES

1. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin 1999;49:33-64.
2. Zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92:690-8.
3. Clarke B, Chetty R. Cell cycle aberrations in the pathogenesis of squamous cell carcinoma of the uterine cervix. Gynecol Oncol 2001;82:238-46.
4. Milde-Langosch K, Riethdorf S. Role of cell-cycle regulatory proteins in gynecological cancer. J Cell Physiol 2003;196:224-44.
5. Funk JO. Cancer cell cycle control. Anticancer Res 1999; 19:4772-80.
6. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501-12.
7. Semczuk A, Jakowicki JA. Alterations of pRb1-cyclin D1-cdk4/6-$p16^{INK4A}$ pathway in endometrial carcinogenesis. Cancer Lett 2004;203:1-12.
8. Murphy N, Heffron CC, King B, Ganuguapati UG, Ring M, McGuinness E, *et al.* $p16^{INK4A}$ positivity in benign, premalignant and malignant cervical glandular lesions: a potential diagnostic problem. Virchows Arch 2004; 445:610-5.
9. Kim JR, Kim SY, Kim MJ, Kim JH. Alterations of CDKN2 (MTS1/p16INK4A) gene in paraffin-embedded tumor tissues of human stomach, lung, cervix and liver cancers. Exp Mol Med 1998;30:109-14.
10. Dong SM, Kim HS, Rha SH, Sidransky D. Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. Clin Cancer Res 2001;7:1982-6.
11. Nuovo GJ, Plaia TW, Belinsky SA, Baylin SB, Herman JG. In situ detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis. Proc Natl Acad Sci USA 1999;96:12754-9.
12. Tsuda H, Hashiguchi Y, Nishimura S, Kawamura N, Inoue T, Yamamoto K. Relationship between HPV typing and abnormality of G1 cell cycle regulators in cervical neoplasm. Gynecol Oncol 2003;91:476-85.
13. Hirama T, Miller CW, Wilczynski SP, Koeffler HP. p16 (CDKN2/cyclin-dependent kinase-4 inhibitor/multiple tumor suppressor-1) gene is not altered in uterine cervical carcinomas or cell lines. Mod Pathol 1996;9:26-31.
14. Kelley MJ, Otterson GA, Kaye FJ, Popescu NC, Johnson BE, Dipaolo JA. CDKN2 in HPV-positive and HPV-negative cervical-carcinoma cell lines. Int J Cancer 1995;

63:226-30.
15. Sano T, Masuda N, Oyama T, Nakajima T. Overexpression of p16 and p14ARF is associated with human papillomavirus infection in cervical squamous cell carcinoma and dysplasia. Pathol Int 2002;52:375-83.
16. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, *et al.* Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001;92:276-84.
17. Marjoniemi VM. Immunohistochemistry in gynaecological pathology: a review. Pathology 2004;36:109-19.
18. Volgareva G, Zavalishina L, Andreeva Y, Frank G, Krutikova E, Golovina D, *et al.* Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. BMC Cancer 2004;4:58.
19. Giarre M, Caldeira S, Malanchi I, Ciccolini F, Leao MJ, Tommasino M. Induction of pRb degradation by the human papillomavirus type 16 E7 protein is essential to efficiently overcome p16INK4a-imposed G1 cell cycle Arrest. J Virol 2001;75:4705-12.
20. Von Knebel Doeberitz M. New molecular tools for efficient screening of cervical cancer. Dis Markers 2001; 17:123-8.
21. Murphy N, Ring M, Killalea AG, Uhlmann V, O'Donovan M, Mulcahy F, *et al.* p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. J Clin Pathol 2003;56: 56-63.
22. Guo M, Hu L, Baliga M, He Z, Hughson MD. The predictive value of p16(INK4a) and hybrid capture 2 human papillomavirus testing for high-grade cervical intraepithelial neoplasia. Am J Clin Pathol 2004;122: 894-901.
23. Saqi A, Pasha TL, McGrath CM, Yu GH, Zhang P, Gupta P. Overexpression of p16INK4A in liquid-based specimens (SurePath) as marker of cervical dysplasia and neoplasia. Diagn Cytopathol 2002;27:365-70.
24. Hu L, Guo M, He Z, Thornton J, McDaniel LS, Hughson MD. Human papillomavirus genotyping and p16INK4a expression in cervical intraepithelial neoplasia of adolescents. Mod Pathol 2005;18:267-73.
25. Fu M, Wang C, Li Z, Sakamaki T, Pestell RG. Minireview: Cyclin D1: normal and abnormal functions. Endocrinology 2004;145:5439-47.
26. Reutens AT, Fu M, Wang C, Albanese C, McPhaul MJ, Sun Z, *et al.* Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/ CBP-associated factor (P/CAF)-dependent manner. Mol Endocrinol 2001;15:797-811.
27. Wang C, Pattabiraman N, Zhou JN, Fu M, Sakamaki T, Albanese C, *et al.* Cyclin D1 repression of peroxisome proliferator-activated receptor gamma expression and transactivation. Mol Cell Biol 2003;23:6159-73.
28. Cho NH, Kim YT, Kim JW. Correlation between G1 cyclins and HPV in the uterine cervix. Int J Gynecol Pathol 1997;16:339-47.
29. Bae DS, Cho SB, Kim YJ, Whang JD, Song SY, Park CS, *et al.* Aberrant expression of cyclin D1 is associated with poor prognosis in early stage cervical cancer of the uterus. Gynecol Oncol 2001;81:341-7.
30. Southern SA, Herrington CS. Differential cell cycle regulation by low- and high-risk human papillomaviruses in low-grade squamous intraepithelial lesions of the cervix. Cancer Res 1998;58:2941-5.
31. Nichols GE, Williams ME, Gaffey MJ, Stoler MH. Cyclin D1 gene expression in human cervical neoplasia. Mod Pathol 1996;9:418-25.
32. Kurzrock R, Ku S, Talpaz M. Abnormalities in the PRAD1 (CYCLIN D1/BCL-1) oncogene are frequent in cervical and vulvar squamous cell carcinoma cell lines. Cancer. 1995;75:584-90.
33. Skomedal H, Kristensen GB, Lie AK, Holm R. Aberrant expression of the cell cycle associated proteins TP53, MDM2, p21, p27, CDK4, cyclin D1, RB, and EGFR in cervical carcinomas. Gynecol Oncol 1999;73:223-8.
34. Cheung TH, Yu MM, Lo KW, Yim SF, Chung TK, Wong YF. Alteration of cyclin D1 and CDK4 gene in carcinoma of uterine cervix. Cancer Lett 2001;166:199-206.
35. Psyrri A, DeFilippis RA, Edwards AP, Yates KE, Manuelidis L, DiMaio D. Role of the retinoblastoma pathway in senescence triggered by repression of the human papillomavirus E7 protein in cervical carcinoma cells. Cancer Res 2004;64:3079-86.
36. Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. Genes Dev 2004;18:2699-711.
37. Grana X, Reddy EP. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 1995;11:211-9.
38. Yoshinouchi M, Hongo A, Takamoto N, Ono Y, Nagao S, Miyagi Y, *et al.* Alteration of the CDKN2/p16 gene is not required for HPV positive uterine cervical cancer cell lines. Int J Oncol 2000;16:534-41.
39. Matsuura I, Denissova NG, Wang G, He D, Long J, Liu F. Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature 2004;8,430:226-31.
40. Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. Cell 1994;78:67-74.
41. Hall M, Bates S, Peters G. Evidence for different modes of action of cyclin-dependent kinase inhibitors: p15 and p16 bind to kinases, p21 and p27 bind to cyclins. Oncogene 1995;11:1581-8.
42. Lukas J, Muller H, Bartkova J, Spitkovsky D, Kjerulff AA, Jansen-Durr P, *et al.* DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. J Cell Biol 1994;125:625-38.
43. Salcedo M, Taja L, Utrera D, Chavez P, Hidalgo A, Perez C, *et al.* Changes in retinoblastoma gene expression during cervical cancer progression. Int J Exp Pathol 2002;83:275-86.
44. Muller H, Lukas J, Schneider A, Warthoe P, Bartek J, Eilers M, *et al.* Cyclin D1 expression is regulated by the

retinoblastoma protein. Proc Natl Acad Sci USA 1994; 91:2945-9.
45. Dowdy SF, Hinds PW, Louie K, Reed SI, Arnold A, Weinberg RA. Physical interaction of the retinoblastoma protein with human D cyclins. Cell 1993;73:499-511.
46. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T. Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. Pathol Int 1998;48:580-5.
47. Amortegui AJ, Meyer MP, Elborne VL, Amin RM. p53, retinoblastoma gene product, and cyclin protein expression in human papillomavirus virus DNA-positive cervical intraepithelial neoplasia and invasive cancer. Mod Pathol 1995;8:907-12.
48. Choo KB, Chong KY. Absence of mutation in the p53 and the retinoblastoma susceptibility genes in primary cervical carcinomas. Virology 1993;193:1042-6.
49. Chetty R, Bramdev A, Aguirre-Arteta A, Pegoraro RJ, Sataar N. Relation between retinoblastoma and p53 proteins in human papilloma viruses 16/18 positive and negative cancers of the uterine cervix. J Clin Pathol 1997;50:413-6.
50. Scheffner M, Munger K, Byrne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci USA 1991;88:5523-7.
51. Fiedler M, Muller-Holzner E, Viertler HP, Widschwendter A, Laich A, Pfister G, *et al.* High level HPV-16 E7 oncoprotein expression correlates with reduced pRb-levels in cervical biopsies. FASEB J 2004;18:1120-2.
52. El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, *et al.* WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994;54:1169-74.
53. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Res 1994;54:3391-5.
54. Lie AK, Skarsvag S, Skomedal H, Haugen OA, Holm R. Expression of p53, MDM2, and p21 proteins in high-grade cervical intraepithelial neoplasia and relationship to human papillomavirus infection. Int J Gynecol Pathol 1999;18:5-11.
55. Kim YT, Cho NH, Park SW, Kim JW. Underexpression of cyclin-dependent kinase (CDK) inhibitors in cervical carcinoma. Gynecol Oncol 1998;71:38-45.
56. Huang LW, Chou YY, Chao SL, Chen TJ, Lee TT. p53 and p21 expression in precancerous lesions and carcinomas of the uterine cervix: overexpression of p53 predicts poor disease outcome. Gynecol Oncol 2001;83:348-54.
57. Van de Putte G, Holm R, Lie AK, Trope CG, Kristensen GB. Corrigendum to "Expression of p27, p21, and p16 protein in early squamous cervical cancer and its relation to prognosis". Gynecol Oncol 2004;92:730.
58. Giannoudis A, Herrington CS. Differential expression of p53 and p21 in low grade cervical squamous intraepithelial lesions infected with low, intermediate, and high risk human papillomaviruses. Cancer 2000;89: 1300-7.
59. Werness BA, Wang HQ, Chance J, Goldstein DJ. p53-independent expression of $p21^{WAF1}$/Cip1 in preinvasive and invasive squamous neoplasms of the uterine cervix. Mod Pathol 1997;10:578-84.
60. Lu X, Toki T, Konishi I, Nikaido T, Fujii S. Expression of $p21^{WAF1}$/CIP1 in adenocarcinoma of the uterine cervix: a possible immunohistochemical marker of a favorable prognosis. Cancer 1998;82:2409-17.
61. Graflund M, Sorbe B, Karlsson M. Immunohistochemical expression of p53, bcl-2, and p21(WAF1/CIP1) in early cervical carcinoma: correlation with clinical outcome. Int J Gynecol Cancer 2002;12:290-8.
62. Cheung TH, Lo KW, Yu MM, Yim SF, Poon CS, Chung TK, *et al.* Aberrant expression of p21(WAF1/CIP1) and p27(KIP1) in cervical carcinoma. Cancer Lett 2001;172: 93-8.
63. Sgambato A, Cittadini A, Faraglia B, Weinstein IB. Multiple functions of p27(KIP1) and its alterations in tumor cells: a review. J Cell Physiol 2000;183:18-27.
64. Shiozawa T, Shiohara S, Kanai M, Konishi I, Fujii S, Nikaido T. Expression of the cell cycle regulator p27 (KIP1) in normal squamous epithelium, cervical intraepithelial neoplasia, and invasive squamous cell carcinoma of the uterine cervix. Immunohistochemistry and functional aspects of p27(KIP1). Cancer 2001;92:3005-11.
65. Huang LW, Chao SL, Hwang JL, Chou YY. Down-regulation of p27 is associated with malignant transformation and aggressive phenotype of cervical neoplasms. Gynecol Oncol 2002;85:524-8.
66. Goff BA, Sallin J, Garcia R, VanBlaricom A, Paley PJ, Muntz HG. Evaluation of p27 in preinvasive and invasive malignancies of the cervix. Gynecol Oncol 2003;88: 40-4.
67. Kim YT, Choi EK, Cho NH, Ko JH, Yang WI, Kim JW, *et al.* Expression of cyclin E and $p27^{KIP1}$ in cervical carcinoma. Cancer Lett 2000;153:41-50.
68. Troncone G, Vetrani A, de Rosa G, Gerbasio D, Palombini L. Cyclin dependent kinase inhibitor $p27^{KIP1}$ expression in normal and neoplastic cervical epithelium. J Clin Pathol 1999;52:880-7.
69. Sgambato A, Zannoni GF, Faraglia B, Camerini A, Tarquini E, Spada D, *et al.* Decreased expression of the CDK inhibitor $p27^{KIP1}$ and increased oxidative DNA damage in the multistep process of cervical carcinogenesis. Gynecol Oncol 2004;92:776-83.
70. Dellas A, Schultheiss E, Leivas MR, Moch H, Torhorst J. Association of $p27^{KIP1}$, cyclin E and c-myc expression with progression and prognosis in HPV-positive cervical neoplasms. Anticancer Res 1998;18:3991-8.
71. Erlandsson F, Wahlby C, Ekholm-Reed S, Hellstrom AC, Bengtsson E, Zetterberg A. Abnormal expression pattern of cyclin E in tumour cells. Int J Cancer 2003; 104:369-75.
72. Geng Y, Yu Q, Whoriskey W, Dick F, Tsai KY, Ford HL, *et al.* Expression of cyclins E1 and E2 during mouse

development and in neoplasia. Proc Natl Acad Sci USA 2001;98:13138-43.
73. Dirks PB, Rutka JT. Current concepts in neuro-oncology: the cell cycle－a review. Neurosurgery 1997;40: 1000-15.
74. Quade BJ, Park JJ, Crum CP, Sun D, Dutta A. *In vivo* cyclin E expression as a marker for early cervical neoplasia. Mod Pathol 1998;11:1238-46.
75. Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Durr P. Inactivation of the CDK inhibitor $p27^{KIP1}$ by the human papillomavirus type 16 E7 oncoprotein. Oncogene 1996;13:2323-30.
76. Southern SA, McDicken IW, Herrington CS. Loss of cytokeratin 14 expression is related to human papillomavirus type and lesion grade in squamous intraepithelial lesions of the cervix. Hum Pathol 2001;32:1351-5.
77. Southern SA, McDicken IW, Herrington CS. Evidence for keratinocyte immortalization in high-grade squamous intraepithelial lesions of the cervix infected with high-risk human papillomaviruses. Lab Invest 2000;80: 539-44.
78. Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D, *et al.* Ki-67, cyclin E, and $p16^{INK4}$ are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol 2001; 25:884-91.
79. Kanai M, Shiozawa T, Xin L, Nikaido T, Fujii S. Immunohistochemical detection of sex steroid receptors, cyclins, and cyclin-dependent kinases in the normal and neoplastic squamous epithelia of the uterine cervix. Cancer 1998;82:1709-19.
80. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. Oncogene 2004;23:2825-37.
81. Dulic V, Lees E, Reed SI. Association of human cyclin E with a periodic G1-S phase protein kinase. Science 1992;257:1958-61.
82. Harbour JW, Dean DC. Rb function in cell-cycle regulation and apoptosis. Nat Cell Biol 2000;2:E65-7.
83. Jones DL, Alani RM, Munger K. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. Genes Dev 1997;11:2101-11.
84. Tetsu O, McCormick F. Proliferation of cancer cells despite CDK2 inhibition. Cancer Cell 2003;3:233-45.
85. Knockaert M, Greengard P, Meijer L. Pharmacological inhibitors of cyclin-dependent kinases. Trends Pharmacol Sci 2002;23:417-25.
86. Kanao H, Enomoto T, Ueda Y, Fujita M, Nakashima R, Ueno Y, *et al.* Correlation between p14(ARF)/p16 (INK4A) expression and HPV infection in uterine cervical cancer. Cancer Lett 2004;213:31-7.
87. Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 1995;83:993-1000.
88. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 1998;92:725-34.
89. Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, *et al.* The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J 1998;17:5001-14.
90. Milde-Langosch K, Riethdorf S, Kraus-Poppinghaus A, Riethdorf L, Loning T. Expression of cyclin-dependent kinase inhibitors $p16^{MTS1}$, $p21^{WAF1}$, and $p27^{KIP1}$ in HPV-positive and HPV-negative cervical adenocarcinomas. Virchows Arch 2001;439:55-61.
91. Brooks LA, Sullivan A, O'Nions J, Bell A, Dunne B, Tidy JA, *et al.* E7 proteins from oncogenic human papillomavirus types transactivate p73: role in cervical intraepithelial neoplasia. Br J Cancer 2002;86:263-8.
92. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, *et al.* Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997;90:809-19.
93. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992;69:1237-45.
94. Chen J, Marechal V, Levine AJ. Mapping of the p53 and mdm-2 interaction domains. Mol Cell Biol 1993; 13:4107-14.
95. Dellas A, Schultheiss E, Almendral AC, Gudat F, Oberholzer M, Feichter G, *et al.* Altered expression of mdm-2 and its association with p53 protein status, tumor-cell-proliferation rate and prognosis in cervical neoplasia. Int J Cancer 1997;74:421-5.
96. Cheng YT, Li YL, Wu JD, Long SB, Tzai TS, Tzeng CC, *et al.* Overexpression of MDM-2 mRNA and mutation of the p53 tumor suppressor gene in bladder carcinoma cell lines. Mol Carcinog 1995;13:173-81.
97. Skomedal H, Kristensen GB, Nesland JM, Borresen-Dale AL, Trope C, Holm R. TP53 alterations in relation to the cell cycle-associated proteins p21, cyclin D1, CDK4, RB, MDM2, and EGFR in cancers of the uterine corpus. J Pathol 1999;187:556-62.
98. Ikenberg H, Matthay K, Schmitt B, Bauknecht T, Kiechle-Schwarz M, Goppinger A, *et al.* p53 mutation and MDM2 amplification are rare even in human papillomavirus-negative cervical carcinomas. Cancer 1995; 76:57-66.
99. Troncone G, Martinez JC, Palombini L, De Rosa G, Mugica C, Rodriguez JA, *et al.* Immunohistochemical expression of mdm2 and $p21^{WAF1}$ in invasive cervical cancer: correlation with p53 protein and high risk HPV infection. J Clin Pathol 1998;51:754-60.
100. Oka K, Suzuki Y, Nakano T. Expression of p27 and p53 in cervical squamous cell carcinoma patients treated with radiotherapy alone: radiotherapeutic effect and prognosis. Cancer 2000;88:2766-73.

101. Busby-Earle RM, Steel CM, Williams AR, Cohen B, Bird CC. p53 mutations in cervical carcinogenesis—low frequency and lack of correlation with human papillomavirus status. Br J Cancer 1994;69:732-7.
102. Milde-Langosch K, Albrecht K, Joram S, Schlechte H, Giessing M, Loning T. Presence and persistence of HPV infection and p53 mutation in cancer of the cervix uteri and the vulva. Int J Cancer 1995;63:639-45.
103. Horner SM, DeFilippis RA, Manuelidis L, DiMaio D. Repression of the human papillomavirus E6 gene initiates p53-dependent, telomerase-independent senescence and apoptosis in HeLa cervical carcinoma cells. J Virol 2004;78:4063-73.
104. Helland A, Holm R, Kristensen G, Kaern J, Karlsen F, Trope C, *et al.* Genetic alterations of the TP53 gene, p53 protein expression and HPV infection in primary cervical carcinomas. J Pathol 1993;171:105-14.
105. Benjamin I, Saigo P, Finstad C, Takahashi H, Federici M, Rubin SC, *et al.* Expression and mutational analysis of p53 in stage IB and IIA cervical cancers. Am J Obstet Gynecol 1996;175:1266-71.
106. Avall-Lundqvist EH, Silfversward C, Aspenblad U, Nilsson BR, Auer GU. The impact of tumour angiogenesis, p53 overexpression and proliferative activity (MIB-1) on survival in squamous cervical carcinoma. Eur J Cancer 1997;33:1799-804.
107. Cho NH, Lim SY, Kim YT, Kim D, Kim YS, Kim JW. G2 checkpoint in uterine cervical cancer with HPV 16 E6 according to p53 polymorphism and its screening value. Gynecol Oncol 2003;90:15-22.
108. Nurse P. Ordering of S phase and M phase in the cell cycle. Cell 1994:79:547-50.
109. Zehbe I, Ratsch A, Alunni-Fabbroni M, Burzlaff A, Bakos E, Durst M, *et al.* Overriding of cyclin-dependent kinase inhibitors by high and low risk human papillomavirus types: evidence for an *in vivo* role in cervical lesions. Oncogene 1999;18:2201-11.
110. Theron T, Bohm L. Influence of the G2 cell cycle block abrogator pentoxifylline on the expression and subcellular location of cyclin B1 and p34cdc2 in HeLa cervical carcinoma cells. Cell Prolif 2000;33:39-50.
111. Hashiguchi Y, Tsuda H, Nishimura S, Inoue T, Kawamura N, Yamamoto K. Relationship between HPV typing and the status of G2 cell cycle regulators in cervical neoplasia. Oncol Rep 2004;12:587-91.