Induction of p16 during immortalization by HPV 16 and 18 and not during malignant transformation

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Summary The p16 (MTS1) tumour-suppressor gene is a cyclin-dependent kinase (cdk) inhibitor that decelerates the cell cycle by inactivating the cdkks that phosphorylate the retinoblastoma tumour-suppressor gene (Rb) protein (pRb). In cervical cancers, pRb is inactivated by the HPV E7 oncoprotein or by mutations. The hypothesis of earlier reports was that the disruption of the p16/cdk-cyclin/Rb cascade is essential for malignant cervical transformation/carcinogenesis. We previously established in vitro model systems of cervical cancer representing four steps of oncogenic progression initiated by the two most common oncogenic HPVs in ectocervical and endocervical epithelial cells. This report used these systems to investigate the role of p16 in cervical cancers. A dramatic enhancement of the p16 RNA level was observed after immortalization by HPV 16 or 18. Furthermore, the p16 protein was newly observed following immortalization. However, no further changes were found for RNA or protein levels after serum selection or malignant transformation. For three cervical carcinoma cell lines, similar high levels of p16 expression were seen. Point mutations or homozygous deletions of p16 were not observed in the in vitro systems or in clinical specimens. These results suggest that the inactivation of the p16/cdk-cyclin/Rb cascade does not occur during malignant transformation but occurs during the immortalization by HPV in HPV-harbouring premalignant lesions, the in situ equivalent of immortalized cells. Also suggested is that p16 has no role in the specific malignant transformation step from immortal premalignant lesions during the carcinogenesis of HPV-initiated cervical cancers.

Keywords: p16; transformation; cervical cancer; immortalization; human papillomavirus; cell cycle; cyclin-dependent kinase; retinoblastoma tumour suppressor

Eukaryotic cell division is regulated by a series of protein kinase complexes consisting of cyclin-dependent kinase (cdk) catalytic units and cyclin control units (Liu et al., 1995; reviewed in Kamb, 1995; Weinberg, 1995). Furthermore, several cyclin-dependent kinase inhibitors have been identified as the control system that prevents the progression of the cell cycle. Examples are p21WAF1 (El-Deiry et al., 1993; Gu et al., 1993; Xiong et al., 1993; Noda et al., 1994), p27 (Polyak et al., 1994; Toyoshiba and Hunter, 1994), p15 (Hannon and Beach, 1994) and p18 (Guan et al., 1994). The deregulation of the cell cycle was proposed as an important mechanism for the malignant transformation of normal cells (Pardee, 1989). For several kinds of cancers, the contribution of mutations in the sequences of the p16 (MTS1) tumour-suppressor gene to the process of the carcinogenesis was proposed. Then, a number of studies showed that point mutations and homozygous deletions of p16 for some types of cancers and cancer cell lines were frequent (Caldas et al., 1994; Guan et al., 1994; reviewed in Kamb, 1995; Liu et al., 1995). However, alterations of p16 were reported to be rare events in some cancers and tumour cell lines (Beltinger et al., 1995; Liu et al., 1995; Quesnel et al., 1995), including cervical carcinomas and cell lines (Kelley et al., 1995; Hirama et al., 1996). Therefore, the significance of p16 in the evolution of cervical cancer remains uncertain.

Cervical cancer is the most frequent cancer of the female genital tract. Cervical carcinogenesis has been studied extensively by various approaches, which firmly established the significance of infections by the oncogenic human papillomaviruses (HPVs) as initiators for the early stages (Zur Hausen, 1994; Zur Hausen and de Villiers, 1994). The role played by promoting cofactor(s) in the later steps of progression to cervical malignancy following initiation remains uncertain, although many candidates are proposed (Herrington, 1995).

Previously, we developed in vitro model systems for cervical cancer. The systems were derived from: the human ectocervix that supports infection; the endocervical origin of most cancers (Tsutsuki et al., 1992); cells immortalized by HPV 16 and 18 that resemble premalignant lesions (Tsutsuki et al., 1992; Yokoyama et al., 1994); immortalized cells adapted to serum that are propagated using the culture conditions used for cervical carcinoma cells (Nakao et al., 1996); and cells malignant transformed by treatment with cigarette smoke condensate (CSC), an HPV co-factor (Yang et al., 1996a; Nakao et al., 1996). The four stages of malignant progression were proposed to represent the in vivo equivalents in the multistep carcinogenesis of cervical cancer. In this report, we examined the RNA, protein and DNA of the important cellular p16 gene for the effects on transformation in our in vitro systems. For comparison, the status of p16 was assessed in biopsies of normal tissues, premalignant lesions and tumours from the cervix.

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**Table 1** Description of cells and cell lines

| Cells       | HPV+ | Serum+ | Tumorigenicity |
|-------------|------|--------|---------------|
| HEN         | -    | -      | NT            |
| HEC         | -    | -      | NT            |
| HEN-16      | 16   | -      | -             |
| HEN-16S     | 16   | +      | -             |
| HEN-16T     | 16   | +      | +             |
| HEN-16-2    | 16   | -      | -             |
| HEN-16-2S   | 16   | +      | -             |
| HEN-16-2T   | 16   | +      | +             |
| HEC-18-1    | 18   | -      | -             |
| HEC-18-1S   | 18   | +      | -             |
| HEC-18-1CT  | 18   | +      | +             |
| CaSki       | 16   | +      | +             |
| HelA        | 18   | +      | +             |
| C33A        | -    | +      | +             |

*HPV type with (−) indicating none. *Adapted to growth in medium with serum (+) or serum-free medium (−). *Tumorigenicity in nude mice was examined. NT, not tested.

**MATERIALS AND METHODS**

**Cells and cell culture**

The cells used in this study are described in Table 1. One series of the stages of carcinogenesis was represented by primary human ectocervical cells (HEC), HPV 18-immortalized HEC (HEC-18-1), serum-adapted HEC-18-1 (HEC-18-1S) and CSC-transformed HEC-18-1 (HEC-18-1CT). The series was established and cultured as described previously (Nakao et al., 1996). Primary human endocervical cells (HEN), HPV 16-immortalized HEN (HEN-16) and another type of HPV 16-immortalized HEN (HEN-16-2) were established and cultured as described previously (Tatsuiki et al., 1992). Fetal calf serum-adapted HEN-16 (HEN-16S) and HEN-16-2 (HEN-16-2S) were initiated and cultured as described previously for HEC-18-1S (Nakao al., 1996). All three types of serum-adapted cells were non-tumorigenic on nude mice. Malignantly transformed HEN-16 (HEN-16T) and HEN-16-2 (HEN-16-2T) were established and cultured as described previously (Yang al., 1996a). The established cervical carcinoma cell lines, CaSki, HeLa and C33A, were cultured with the same conditions as the tumorigenic immortalized cells. For serum-adapted cells immortalized by HPV 16 and the corresponding malignantly transformed cell lines, the medium was supplemented with 100 ng ml⁻¹ EGF and 0.4 μg ml⁻¹ hydrocortisone.

**Clinical samples**

Paraffin-embedded tissue biopsies of various pathological lesions of the cervix were from the Sir Mortimer B Davis-Jewish Hospital, Montreal, Canada, and from T Wright and R Richart for invasive adenocarcinomas of the endocervix. Samples were examined for the presence of HPV DNA in DNA extracted by a previously described method (Koffa et al., 1995). HPV-negative samples were tested for the integrity of the DNA by polymerase chain reaction (PCR) analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA. Based on the results, 14 samples were selected for further studies of p16. The HPV type and the pathological diagnosis of each sample are shown in Table 2.

**Table 2** List of clinical samples, HPV type and pathological diagnosis

| Sample | HPV type | Diagnosis |
|--------|----------|-----------|
| 1      | –        | NSPAa     |
| 2      | –        | NSPA      |
| 3      | 16       | HGSILd    |
| 4      | –        | HGSIL     |
| 5      | 16       | SCCc      |
| 6      | 16       | SCC       |
| 7      | 16       | SCC       |
| 8      | 16       | SCC       |
| 9      | 16       | SCC       |
| 10     | 18       | SCC       |
| 11     | 16       | Adenocarcinoma |
| 12     | 18       | Adenocarcinoma |
| 13     | 18       | Adenocarcinoma |
| 14     | –        | Adenocarcinoma |

*+, HPV negative. NSPA, no specific pathological alteration. HGSIL, high-grade squamous intraepithelial lesion. SCC, squamous cell carcinoma.

**Northern blot RNA analysis**

Total cellular RNA was prepared and analysed using Northern blot assays as described previously (Yang et al., 1996b). The probe for the complete p16 cDNA was randomly labelled with [³²P]dCTP. The actin probe was used as a control for the amount of RNA loaded and efficiency of blot transfer.

**Polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis of p16 DNA**

To test for sequence alterations of p16, PCR-SSCP analysis was used with DNA from the cells and samples shown in Tables 1 and 2. The three primer pairs are described in Table 3 and were designed previously (Zhang et al., 1994). One pair is for the analysis of p16 exon 1 and the other two pairs are for partly overlapping regions of most of exon 2. Two paraffin-embedded 8-μm slices were extracted as described previously (Koffa et al., 1995). Genomic DNA (50 ng) was amplified in the presence of 1 μCi [³²P]dCTP (New England Nuclear). PCR was as follows: 94°C for 2 min; 10 cycles of 94°C for 1 min and 62°C for 1 min; 72°C for 1 min; and 6 min at 72°C. PCR-amplified DNA was resolved in 6% polyacrylamide sequencing gels at 4°C. The gels were dried without fixation and exposed to Kodak XAR film for 12–72 h. The PCR amplification was repeated twice or more, and the results were consistent.

**HPV typing**

The PCR non-radioactive HPV detection system used HPV L1 open reading frame consensus primers as described previously (Yoshikawa et al., 1991) with some modifications. The system was designed for highly sensitive and reliable screening and typing of HPVs contained in clinical samples. This system can identify a minimum of nine genital HPV types, HPV 6, 11, 16, 18, 31, 33, 42, 52 and 58. Amplification of 50 ng of DNA from paraffin-embedded tissue was as follows: 94°C for 2 min; 40 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min; and 6 min at 72°C. Electrophoresis of amplified DNA was in 1.5–3% agarose...
Table 3 Sequence and experimental design of primers

| Sequence                      | Experimental design          |
|-------------------------------|------------------------------|
| p16 E1-sense, 5'-GCTCGGAGAGGGGAGAGCAGGCA-3' | SSCP‡ for exon 1            |
| p16 E1-antisense, 5'-GCACTGCTGTACCCAATTTC-3' | SSCP for exon 1             |
| p16 E2-1-sense, 5'-ACAAGCTTCTTTCCGTCATGC-3' | SSCP for exon 2-1           |
| p16 E2-antisense, 5'-TTCTGGAACACGCTGTTG-3' | SSCP for exon 2-2           |
| p16 E2-antisense, 5'-TCTGAGCTTTGGAAGCTCTCAG-3' | SSCP for exon 2-2           |
| HPV L1-sense, 5'-CGTAAACGTTTCCCCATTTTTTTTTT-3' | HPV screening               |
| HPV L1-antisense, 5'-TACCAAAATCTGCTAT-3' | HPV screening               |
| GAPDH-sense, 5'-AGTACGCTGAGGGCCTACTCCT-3' | Control for DNA quality     |
| GAPDH-antisense, 5'-AAGAGCCAGTCTCTGCGCCCAGCCA-3' | Control for DNA quality     |

‡SSCP, single-strand conformation polymorphism.

gels. Positive samples were examined by restriction enzyme analysis to identify the HPV type. PCR analyses were repeated twice or more and were consistent. For HPV-negative samples, the integrity of the DNA was tested by PCR analysis for GAPDH as a control, using the same conditions as for p16.

Western blot analysis

Protein was extracted from approximately 10⁷ growing cells by lysis in 1 ml of 0°C extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.02% sodium azide, 1% NP-40, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate and 10 µg ml⁻¹ aprotinin) for 30 min and centrifuging at 12 000 g for 10 min at 4°C. The DC protein assay kit (Bio-Rad) was used to quantify the protein. Ten micrograms of protein were fractionated by electrophoresis in 15% SDS-polyacrylamide gels according to standard protocols. Stained protein markers (Amersham) were included in each gel as molecular weight standards. The proteins were subsequently transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane under semi-dry conditions. For this non-radioactive analysis method, immunodetection of p16 polyclonal antibody (C-20) (Santa Cruz) at 1:100 dilution was performed using the ECL system (Amersham) according to the manufacturer’s instructions.

RESULTS

Expression of p16 RNA in in vitro model of cervical carcinogenesis

Our in vitro model systems were used to examine the level of transcriptional expression of p16 in four stages of malignant progression by Northern blot analysis for the first 11 cell types listed in Table 1. Representing the first stage were the human primary endocervical cells, HEN. The second stage comprised two lines of HPV 16-immortalized human endocervical cell lines, HEN-16 and HEN-16-2. Third, these lines were used to produce the serum-adapted non-tumorigenic cells, HEN-16S and HEN-16-2T. The fourth and final stage was represented by the tumorigenic clones, HEN-16T and HEN-16-2T. The analogous stages were represented by the following: primary human ectocervical cells, HEC; one line of HPV 18-immortalized derived cells, HEC-18-1; the serum-adapted non-tumorigenic derivative cells, HEC-18-1S; and the tumorigenic counterpart cells, HEC-18-1CT. These cells of the model systems were compared with three cell lines of established human cervical cancer cells: CaSk containing HPV 16, HeLa containing HPV 18 and C33A containing no HPV.

In Northern blot analysis, the transcriptional expression of p16 was undetectable in primary HEN (Figure 1A) and very low in HEC (Figure 1B). The RNA level was dramatically enhanced following immortalization by HPV. The up-regulation was observed irrespective of the type of HPV. Further, the results were qualitatively similar for both HEN-16- and HEN-16-2-immortalized HEN lines, representing the second stage in progression to malignancy. Serum-adapted but non-tumorigenic HPV-immortalized cells were used for the next stage in progression, as adaptation to serum is correlated with the expression of growth-related genes (Rossi and Hirschhorn, 1991). In addition, the cells served as controls for the effect of the growth conditions used in generating and propagating the tumorigenic cells representing the final stage in malignant progression. Relative to the actin control, no effects of serum selection were seen. More importantly, tumorigenic cells malignantly transformed by the HPV cofactor in cervical carcinogenesis, CSC, were examined. Compared with the other two stages of HPV-immortalized cells, they showed no significant alteration in the level of transcription. Again, this result applied to the three lines, both HPVs and two different cervical cell types. To test the relevance to naturally occurring cervical cancer, the established cervical carcinoma cell lines C33A, CaSk and HeLa were examined. All three showed the same high expression level of the p16 tumour-suppressor gene as the three stages of HPV-immortalized cells. These levels were much higher than for HEC and HEN (Figure 1A and B).

Level of p16 protein in in vitro model

To test the possible effect of post-transcriptional or translational regulation of p16, we used Western blot analysis with same model systems for malignant progression. The expression pattern of p16 protein was consistent with the results of mRNA expression. In comparison with the HEN human primary endocervical cells (Figure 2A) and HEC ectocervical parental cells (Figure 2B), the expression of p16 was enhanced in the HPV 16- and 18-immortalized cells. The level of expression was stable during the final three steps of carcinogenesis of the HPV-immortalized cells, regardless of the changes that occurred during serum adaptation and tumorigenesis (Yang et al, 1996a; Nakao et al, 1996). The three lines of established cervical cancer cells showed high expression levels of p16, protein levels consistent with those of our experimental model systems (Figure 2).
Point mutations and homozygous deletions in p16 for model for oncogenesis

To determine whether mutations of p16 were involved in the three steps of transformation of our cultured cervical cells, PCR-SSCP analysis for band shifting was performed. One set of primers for exon 1 and two sets for exon 2 of p16 (Table 3) were used in three separate analyses. However, no differences in the pattern of bands were revealed by shifts for the HEN-16-series, HEN-16-2-series and HEC-18-series in vitro models for cervical carcino genesis. Exon 1 and exon 2–1 primers revealed no changes in band patterns (data not shown). Typically, the same result was shown by exon 2–2 (Figure 3). All the cell lines showed a pattern that was identical to those of the parental primary endo- and ectocervical cells, HEN and HEC. Also, none had the complete loss of signals that would indicate homozygous deletions. The results among the HPV-positive HeLa and CaSki and HPV-negative C33A cervical carcinoma cell lines were consistent with our model and with previous reports (Figure 3; Kelley et al, 1995; Hiramta et al, 1996).

DISCUSSION

The p16 gene product inhibits the progression of the cell cycle through G1, by binding to cdk4/6. Thereby, the phosphorylation of the retinoblastoma gene (Rb) protein (pRb) by the cdk5 is prevented. Consequently, pRb binds to and depletes the level of the E2F transcription factor required for the expression of the S-phase oncoproteins that activate cell cycling. Therefore, the loss of functional p16 disrupts the pattern of cellular gene expression and initiates carcinogenesis through the deregulation of the cell cycle (Kamb et al, 1994). Other recent information also indicates that
\( p16 \) functions as a negative regulator of the cell cycle in the presence of wild-type Rb through the \( p16/cdk\)-cyclin/Rb cascade (Guan et al., 1994; Lukas et al., 1995). In cells containing an inactivated Rb or harbouring specific DNA tumour viruses, E2F is not sequestered by Rb, resulting in uncontrolled cell growth (Aagaard et al., 1995; Whitaker et al., 1995; Yeager et al., 1995). Furthermore, wild-type pRb down-regulated the promoter of \( p16 \), whereas the negative feedback by low levels of p16 enhanced \( p16 \) expression in Rb-inactivated, HPV-positive cells (Li et al., 1994). Recently, the expression of \( p16 \) was found to be inversely correlated with pRb and Bcl-2 in cancer cell lines and cancers (Otterson et al., 1994; Nakamura et al., 1996; Sakaguchi et al., 1996). For example, the C33A used in this study have no functional Rb. The results with \( p16 \) indicate the importance of \( p16 \) to the in vivo condition. The overexpression of \( p16 \) was observed in HPV-positive cell lines and cell lines expressing mutant Rb (Tam et al., 1994; Aagaard et al., 1995). Although it would be of further interest to test \( p16 \) expression in cervical tumour, these data are consistent with the hypothesis that \( p16 \) expression and function are dependent on Rb.

In cervical carcinogenesis, most cancers contain oncogenic HPV DNA (Bosch et al., 1995). In vitro, the expression of viral E7 was necessary for transformation (von Knebel-Doeberitz et al., 1988). E7 binds and inactivates pRb. The introduction of HPV during cervical cancer development apparently acted on the \( p16/cdk\)-cyclin/Rb cascade by inactivating pRb, the \( p16 \) downstream alternate target. However, it was unknown whether changes during tumorigenesis were involved. Our in vitro model systems suggest that the inactivation of pRb by HPV E7 occurred in the initial immortalization step of oncogenesis. Consistently, no changes in levels of HPV mRNA were found during tumorigenesis in these systems previously (Nakao et al., 1996; Sarma et al., 1996). No changes in expression level or genetic alterations of \( p16 \) were suggested to be involved in tumorigenesis after immortalization. This hypothesis is consistent with previous reports that genetic alterations of \( p16 \) were rarely seen in HPV-harbouring cervical carcinoma cell lines and HPV-negative cell lines that contain mutant Rb (Kelley et al., 1995; Hiramatsu et al., 1996).

Our in vitro model systems represent four stages of cervical cancer: first, normal primary human endo- and ectocervical cells.
representing metaplastic and normal cervical tissues respectively. Next, the HPV 16- or HPV 18-immortalized human cervical cells and serum-adapted cells represent the continuum of progression to high-grade squamous intraepithelial lesions (HGSILs). Finally, the malignantly transformed cells represent cervical tumours. The advantages of these systems in investigating multistep carcinogenesis are: (1) the origins of the immortalized cells are clearly defined and distinct — each cell line was clearly endocervical or ectocervical in origin (Tsutsumi et al, 1992; Sun et al, 1993; Yokoyama et al, 1994); (2) immortalized cells were derived with the two HPVVs most frequently associated with the initiation of cervical oncogenesis (zur Hausen, 1994); (3) malignant clones were derived by treatment with CSC — cigarette smoking was proposed as an environmental cofactor of HPV, based on epidemiological and molecular biological studies (Herrington, 1995). Therefore the transformation of HPV-immortalized cervical cells by CSC is relevant to the in vivo situation and provides a suitable in vitro model for cervical cancer (Sarma et al, 1996; Yang et al, 1996, b); and (4) the clinical lesions are relevant for the reasons stated below. Previously, we reported differential oncogenic potentials for our model systems with the in vitro organotypic (raft) culture and in vivo implantation differentiation systems. HPV-immortalized endocervical cells reconstructed into HGSIL-like lesions, and HPV-immortalized ectocervical cells showed low-grade SIL (LGSIL)-like lesions in the two differentiation systems. Cells malignantly transformed by CSC resembled HGSIL-invasive squamous cell carcinomas in the in vitro differentiation system and were tumorigenic on nude mice (Nakao et al, 1996; Yang et al, 1996a). These results show that HPV-immortalized cells correspond to LGSIL-HGSILs (Sun et al, 1992; Tsutsumi et al, 1992; Yokoyama et al, 1994; Nakao et al, 1996; Yang et al, 1996a). Because of these four reasons above, our systems are attractive for studying the progressive stages of cervical cancer.

The role of p16 as a tumour-suppressor gene remains controversial at present. Tumour-suppressor genes can act in early or late stages of carcinoma development (reviewed by Vogelstein and Kinzler, 1993). The roles of HPV E6 and E7 in inactivation of p53 and pRb tumour suppressors are required but not sufficient. For example, p53 deletions are seen in both HPV-positive and -negative cervical cancer (Mullokandov et al, 1996). Thus, the role of pRb in senescence control may not have been its only role. Our experiments support the previous finding that mutations of p16 infrequently disrupt the p16/cdk-cyclin/Rb cascade for the transformation that leads to cervical cancer (Kelley et al, 1995; Hiramia et al, 1996). Enhanced expression of p16 was observed in the initial immortalization step by HPV. Furthermore, our studies in the in vitro systems indicated that no inactivation of the p16/cdk-cyclin/Rb cascade occurs during the serum adaptation and tumorigenesis of HPV-immortalized cells. This cascade was apparently inactivated by the introduction of HPV but was insufficient for progression to the malignant transformation stage. Consistently, previous results suggested that p16 did not contribute to tumorigenesis, as alterations of this gene were already present in non-tumorigenic immortalized breast epithelial cells (Cairns et al, 1994; Spruck et al, 1994; Brenner and Aldaz, 1995).

In cytogenetic studies of cervical cancer, high-frequency loss of heterozygosity (LOH) and deletion of chromosomes 1, 3, 4, 5, 6, 10, 11, 17, 18 and X was reported (Sreekantaiah et al, 1988; Yokota et al, 1989; Misra et al, 1994). A recent small-scale study found LOH of the 9p21 locus to which p16 is mapped in 20% of cervical cancer cases (Cairns et al, 1995). However, the short arm of chromosome 9 or 9p21 have not been generally recognized as ‘hot spots’ for LOH. These findings are consistent with our finding no mutations of p16 in the in vitro model. Further, our examination of clinical samples was useful, although the number was relatively limited and the possible contamination of some samples with normal tissue surrounding the pathological lesions cannot be excluded. However, our results were relevant in showing that mutations of p16 are also infrequent events in clinical cervical cancers.

Despite the absence of p16 mutations in our immortalized cells grown in vitro, RNA and protein levels were enhanced. Other factors, such as mutations of splice sites and promoter sequences, were shown to influence the level of other proteins in various genetic diseases, including cancers (Friedman et al, 1994). Also, events occurring downstream of the p16 DNA may be involved in the altered expression levels. Expression was unaltered following tumorigenesis by CSC. Consistently, no published reports have associated cancers that are promoted by smoke with the expression of p16. Apparently, the dysregulation of the cell cycle by p16 is not the final step in progression to malignancy. Therefore, our results showing increased levels of RNA and protein expression following immortalization are important for clarifying the possible mechanism of initiating events in the carcinogenesis of cervical cancer. The fact that no increase was shown following the ultimate tumorigenesis was also revealing.

In conclusion, we suggest that (a) genetic alterations of p16 are rare events in cervical carcinogenesis; (b) transcription of p16 is the level at which disruption of the p16/cdk-cyclin/Rb cascade occurs; (c) the disruption occurs during HPV-mediated immortalization and HPV infection; (d) smoking is an additional cofactor that acts by mutating the host genome, probably in p16-independent mechanisms, although it is necessary for the full malignant transformation that results in cervical epithelial neoplasms.

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