The role of cyclins and cyclin inhibitors in the multistep process of HPV-associated cervical carcinoma

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Abstract

Background: Human papillomavirus (HPV) types 16 and 18 are associated with cervical carcinogenesis. This is possibly achieved through an interaction between HPV oncogenic proteins and some cell cycle regulatory genes. However, the exact pathogenetic mechanisms are not well defined yet.

Methods: We investigated 110 subjects (43 invasive squamous cell carcinoma [ISCC], 38 CIN III, 11 CIN II, 18 CIN I) confirmed to be positive for HPV16 and/or 18 as well as 20 normal cervical tissue (NCT) samples for abnormal expression of *cyclin D1, cyclin E, CDK4,* cyclin inhibitors *($p21^{waf}$, p27, $p16^{INK4A}$) and Ki-67* using immunohistochemistry and differential PCR techniques.

Results: There was a significant increase in the expression of *Ki-67*, *cyclin E, CDK4, $p16^{INK4A}$ (p*= 0.003**,** 0.001**,** 0.001*)* and a significant decrease in *$p27^{KIP1}$* from NCT to ISCC (p=0.003). There was a significant correlation between altered expression of *$p27^{KIP1}$and $p16^{INK4A}$* (*p<0.001*), *cyclin D1* and *CDK4* (*p*=0.001), *cyclin E* and *$p27^{Kip1}$* (*p*=0.011) in all studied groups. In ISCC, there was significant relationship between standard clinicopathological prognostic factors and high *Ki-67* index , increased *cyclin D1* and *cyclin E,* reduced *$p27^{Kip1}$* and *$p21^{waf}$*.

Conclusion: 1) Aberrations involving *$p27^{KIP1}$*, *cyclin E, CDK4* and *$p16^{INK4A}$* are considered early events in HPV 16 and 18-associated cervical carcinogenesis (CINI&II), whereas *cyclin D1* aberrations are late events (CINIII&ISCC). 2) Immunohistochemical tests for *$p16^{INK4A}$ and cyclin E* could help in early diagnosis of cervical carcinoma. 3) Only FIGO stage*, cyclin D1, $p27^{KIP1}$*and *Ki-67* are independent prognostic factors that might help in predicting outcome of cervical cancer patients.

Introduction

Carcinoma of the uterine cervix emerges from a defined series of prenoplastic lesions with increasing cellular dysplasia referred to as cervical intraepithelial neoplasia (CIN) grade I, II and III (1). A large body of knowledge supports the view that high risk HPV types (HR-HPV) are strongly associated with invasive squamous cell carcinoma (ISCC) and its precursors as they have the ability to transform normal cervical cells into neoplastic ones (2). HPV types 16 and 18 are the most commonly detected HR-HPV types in these lesions, and therefore their detection was proposed as a useful surrogate marker to diagnose cervical dysplasia and carcinoma in situ (CIS) (2, 3).

Progress in cervical cancer research provides evidence that integration of HR-HPV DNA into the host cell genome results in elevated expression levels of the *E6* and *E7* proteins with subsequent interaction between these proteins and the cell cycle machinery (2, 4). Expression of *E6* and *E7* proteins of HR-HPV types induces immortalization of cells through their inhibitory effects on the tumor suppressor proteins *pRb* and *p53*; respectively, altering the cell cycle control and leading to chromosomal instability (2). However the *E6-p53- E7-Rb* model is not sufficient to inevitably produce cervical carcinoma although it has resulted in the identification of the viral gene's actions on numerous cellular proteins and processes normally involved in cellular growth and proliferation. This is evidenced by the spontaneous clearance of HPV infection and the long delay between the onset of persistent infection and the emergence of malignancy.

Recent studies show an association between HR-HPV types and cell cycle regulators (2,5). The cell cycle is governed by a family of *cyclins*, *cyclin dependent kinases (CDKs)* and their inhibitors *(CDKIs)* through activating and inactivating phosphorylation events. Attention has been focused on altered expression of G_1 *cyclins*

and *Cdks* because the major regulatory events leading to cell proliferation and differentiation occur within the G_1 phase of the cell cycle. The D-type *cyclins* reach maximum levels of expression and form functional kinase complexes with *CDK4* or *CDK6;* during the mid-G_1 phase, whereas *cyclin-E* is expressed and associated with *Cdk2* in an active complex near the G_1-S boundary (5, 6). Active *CDK/cyclin* complex could be regulated by binding to *CDKI* ($p16^{INK4A}$, $p21^{waf1}$ and $p27^{KIP1}$) and inhibit cell cycle progression from G1 to S phase (5).

Deregulation of $p16^{INK4A}$, $p21^{waf1}$ and $p27^{KIP1}$ has recently been reported in various human tumors. In HPV-associated cervical carcinoma, the expression and function of these proteins is supposed to be impaired by the action of viral oncopoteins *E6* and *E7* (7). However, it is unclear how and when factors that are innate to the HPV-infected cells including genetic aberrations launch the host cell into an irreversible progression to cancer (5).

Therefore, we attempted to assess the contribution of *cyclin D1, cyclin E, CDK4,* $p21^{waf}$, $p27^{KIP1}$, $p16^{INK4A}$ and *Ki-67* expression to the development of HPV16/18-associated cervical carcinoma and to determine at what stage of carcinogenesis these aberrations start to manifest. The prognostic value of these aberrations was also investigated in relation to the standard clinicopathological prognostic factors.

Material and methods

Tumor samples from HPV positive cervical tissues of different grades, as well as normal cervical tissues were examined for the expression level of *cyclin D1, cyclin E, CDK4,* $p21^{waf}$, $p27^{KIP1}$, $p16^{INK4A}$ and *Ki-67* genes by immunohistochemistry. Whereas, *cyclin D1, cyclin E and CDK4* gene status were determined by differential PCR assay. *p16* gene mutation was also tested. These markers were correlated with standard prognostic factors.

Clinical samples: The study included 110 fresh-frozen tissue samples [43 invasive squamous cell carcinoma [ISCC] and 38 cervical intra-epithelial neoplasia [CIN] III, 11 CINII and 18 CINI]. They were selected from a total of 200 cases based on HPV16 and/or HPV18 positivity. Cases were collected from patients who were diagnosed and treated at the National Cancer Institute (NCI), the hospitals of Ain-Shams University and Kasr El-Aini School of Medicine during March 1999- July 2003 after a recent evidence of abnormal cervical cytology and/or conization biopsy (obtained by large loop excision of the transformation zone).

Cases of ISCC were examined by two independent pathologists, classified and graded according to the World Health Organization (WHO) criteria and staged according to criteria of the International Federation of Gynecolology and Obstetrics (FIGO) (8). The clinicopathological features of the studied cases are illustrated in table 1. Twenty normal cervical tissues (NCT) obtained from patients undergoing hysterectomy for medical conditions not related to the cervix were included in the study as a control group. All control samples showed normal cervical cytology and histology and all were negative for HPV by PCR. In ISCC and CIN samples, only cases with ≥75% tumor cells were included in the study. A written consent was obtained from all patients prior to

enrollment in the study, and the ethical committee of the NCI approved the protocol which was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Cell lines: The HPV16 positive (*SiHa*) and HPV18 positive (*HeLa*) cervical carcinoma cell lines (from Institute of Human Genetics, Academic Medical Centre, Amsterdam, The Netherlands) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma, USA) supplemented with 10% fetal bovine serum (Sigma, USA) and were used as controls.

DNA extraction: High molecular weight DNA was extracted from fresh tumors, normal samples and cell lines according to standard protocols (9).

HPV detection and typing: The purified DNA was subjected to PCR amplification using general-purpose HPV primers (*GP5+ and GP6+)* which amplify conserved sequences in the HPV-L1 region (150 bp) as previously described (10). To determine the HPV type, PCR products were separated by electrophoresis on 1.5% agarose gel, transferred onto a nylon membrane (Hybond N +, Amersham) and analyzed using digoxigenin-labelled type-specific probes by chemiluminescent detection (ELOCA)**.** The HPV probe consisted of a cocktail of 14 high-risk mucosal types [16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 62, 66, and 68] and two low-risk types [6 and 11].

Immunohistochemistry: Tumors and normal tissue samples were put in 10% neutral buffered formalin and processed routinely for preparation of hematoxylin and eosin-stained slides and for immunohistochemical studies. Five micron thick sections were cut onto positive-charged slides and were used for immunohistochemical detection of *cyclin D1, cyclin E, CDK4,* $p21^{waf}$, $p27^{KIP1}$, $p16^{INK4A}$, *and Ki-67.* The standard streptavidin-biotin-peroxidase detection technique was performed using the antibodies illustrated in table 2. Briefly, after deparaffinization in xylene and rehydration through graded

alcohols, sections were microwaved in 0.01M citrate buffer (pH 6.0) for 15min. Endogenous peroxidase activity was blocked by immersion in 0.03% H_2O_2 in methyl alcohol for 30 min and 10% normal rabbit (for mouse primary antibodies) or goat serum (for rabbit primary antibodies) was applied to avoid non-specific reaction. Sections were then incubated with the primary antibodies or with non-immunized mouse or rabbit serum for the negative control at 4^0C. After washing with PBS, biotinylated anti-mouse or rabbit IgG was applied for 30 min at room temperature. The peroxidase-conjugated-streptavidin solution was applied for 30 min and visualized using 0.05% 3'-3' diaminobenzidine (DAB). Counterstaining was performed with Meyer's hematoxylin (11).

Only a distinct brown nuclear staining was scored positive. In NCT: cyclin *D1; cyclin E* and *CDK* were focally expressed in the parabasal cells, $p21^{waf}$ was focally detected and $p27^{KIP1}$ was detected in >50% of the cells. This was the normal expression pattern for the studied protein (6). An aberrant expression of *cyclin D1, cyclin E, CDK4*, $p21^{waf}$ and $p16^{INK4A}$ was defined as staining in excess of normal tissues (> 20% for cyclin *D1; cyclin E, CDK4* and >10% for $p21^{waf}$ *and* $p16^{INK4A}$) (Fig.1) whereas loss of $p27^{KIP1}$ was defined as staining in <50% of the cells (6). A negative control was obtained by replacing the primary antibody by non-immunized rabbit or mouse serum.

Four semiquantitative classes were used to describe the percentage of positively-stained tumor cells: negative (no cells stained); +: minimally positive (1-10% positive cells); ++: moderately positive (10-50% positive cells); and +++: markedly positive (>50% positive cells). The positivity of each stain including *Ki-67* was also described as a positivity index (PI) which indicated the number of positive cells in 1000 arbitrarily selected, manually counted cells at 200X magnification (3, 4,12).

Determination of cyclin D1, cyclin E and CDK4 gene status: This was done using the differential PCR assay (DPA) as previously described (13) with GAPDH as an internal control.

Determination of p16 gene mutation*:* The presence of *p16* gene mutations was assessed using the SSCP/sequencing according to Tripathi et al. (14).

Statistical methods*:* Statistical analysis was performed using the Stat View 4.5 software package (Abacus Concepts, Berkeley, CA). The Mann-Whitney non parametric test was used to compare the PIs of pairs of subjects and the Kruskal-Wallis test was used for categorical data. Correlation between the indices was determined by a simple linear regression test.

Results

The expression level of the studied markers:

Expression of cyclins and CDK: *Cyclin D1* overexpression was detected in CINIII and ISCC cases only. All samples positive for *cyclin D1* expression were scored (++) except for 6 cases of ISCC that scored (+++). The PI was significantly higher in invasive squamous cell carcinoma than in CINIII (p= 0.001). *Cyclin E* expression was significantly increased from CINI (11.1%; PI=18±1.1) to ISCC (88.4%; PI=97±13.8), (p= 0.001). Similarly, *CDK4* expression was significantly increased from CINI to ISCC (p= 0.001) (Figure 1 & Table 3).

Cyclin D1 gene amplification was detected in 20 (46.5%) ISCC and 7 (18.4%) CINIII cases (The concordance between both techniques was 90 %). *Cyclin E* gene amplification was detected in 2 cases (11.1%) of CINI, 5 (45.5%) CINII, 21 (55.3%) CINIII and 38 (88.4%) ISCC (The concordance between both techniques was 97%). *CDK4* gene amplification was detected in 2 cases of CINI, 4 cases of CINII, 15 cases of CINIII and 39 cases of ISCC (The concordance between both techniques was 96%) (Figure 3 a-c).

Expression of CDKIs: Nuclear immunoreactivity for $p21^{waf}$ was sporadically detected in NCT, CINI and CINII. However, diffuse staining for $p21^{waf}$ was detected in CINIII and ISCC. There was a significant difference in the PI of $p21^{waf}$ between CINII and CINIII as well as between CINIII and ISCC (p= 0.01) (Figure 1& Table 3).

$p27^{Kip1}$ expression was significantly decreased with disease progression (p=0.003). The protein was found in all NCT and CINI, in 63.6% of CINII, 26.3% of CINIII and in 13.9%of ISCC (Figure 1 & Table 3).

$p16^{INK4A}$ overexpression was detected in 4 CINII (36.4%), 24 CINIII (63.15%) and 40 ISCC (93.0%). The PI was significantly higher in ISCC than in CINIII (p=0.01) (Figure

1& table 3). Although abnormal conformers were detected in 3 (6.97%) ISCC cases by SSCP, no mutations were found in any of them by sequencing.

Ki-67 expression: The positivity index (PI) for *Ki-67* was significantly increased with progression from NCT (<0.1) to ISCC (40.5±11) (p= 0.003). *Ki-67* expression in CINI was significantly higher than in normal cervical tissues (p=0.01) (Figure 2& Table 3).

Clinical correlations: There was a significant correlation between altered expression of $p27^{KIP1}$ *and* $p16^{INK4A}$ ($p<0.001$), *cyclin D1* and *CDK4* (p=0.001) as well as between increased *cyclin E* and reduced $p27^{Kip1}$ in all studied groups (p=0.011).

In the group of ISCC, there was significant correlation between reduced $p27^{Kip1}$, tumor size, tumor type (p=0.047), positive lymph node, stromal invasion (p=0.001& p=0.024) and FIGO stage (p=0.002); between $p21^{waf}$ overexpression and tumor size (p=0.001) and vaginal involvement (p=0.042). Also, increased *cyclin D1* was significantly associated with tumor size (p=0.003) and FIGO stage (p=0.008) whereas, increased *cyclin E* expression was significantly associated with tumor size (p=0.001). A high *Ki-67* index was significantly associated with tumor size, lymph node involvement and FIGO stage (Table 1).

Discussion

The present study is the first to investigate the role of a large panel of cell cycle regulatory genes in Egyptian patients with HPV-associated cervical carcinoma both at the gene and protein levels.

It has been shown that the E6 and E7 proteins of HR-HPV types disrupt cell cycle checkpoints, particularly affecting *CDKI* linked to the G1- and G2-checkpoints with consequent accumulation of genetic aberrations (**15**)**.** To our knowledge, the role of *cyclins*, *CDKs* and *CDKI* in ISCC and its precursor lesions is not well defined yet. Moreover no single study has investigated the contribution of these proteins together and in association with other cell cycle related genes such as *p53, Rb* and *mdm-2.* In the present study aberrant expression of *CDKs* and their inhibitors were detected at several points during the transformation of HPV-infected cervical epithelium.

C*yclin D1* amplification and protein overexpression were found in 46.5% and 41.9% of our ISCC cases as well as in18.4% and 13.15% of CINIII cases only. Our results are in agreement with Nicholas et al. (**15**) and Cheung et al. (**13**). In contrast, Bae et al. (**16**) reported reduced *cyclin D1* mRNA and protein expression in cases of CIN and ISCC compared to NCT. It is difficult to explain the controversy between our results and those of Bae et al. (**16**)**.** However, their study is one of the very few studies showing reduced *cyclin D1* in cervical carcinoma. Moreover, they reported increased *cyclin D1* expression with increasing severity of the lesion from high grade squamous intraepithelial neoplasia (CINII-III) to ISCC which makes a higher expression in normal epithelium compared to the neoplastic one unlikely.

Our study shows a stepwise increase in the expression level of *CDK4* from normal to tumor tissues indicating an important role for *CDK4* at an early stage of transformation of HPV- infected cervical epithelium. Our results confirm the few

available reports in this context (**13, 6**). Although it is well known that *cyclinD1* is necessary for the activation of *CDK*, the concordance reported in the present study between *CDK4* and *cyclin D1* expression was 49% only. We also detected increased *CDK4* expression in cases of CINI and CINII although these cases did not show simultaneous increase in *cyclin D1*. This could be explained by the involvement of other D-type *cyclins* such as *cyclin D2 and 3* in the activation of *CDK4* (**17**). Alternatively, the D type *cyclins* may not be required at all for G1 progression in HPV-transformed cervical epithelium since binding of HPV E7 protein to *Rb* leads to release of *E2F* transcription factor omitting the role of *cyclin D1* in cell cycle progression (**15**).

Our results denote an important role for *cyclin E* in the early stages of HPV-associated cervical carcinogenesis since protein overexpression and gene amplification were detected during progression from NCT into ISCC. We also noticed a significant association between increased *cyclin E* expression and reduced $p27^{kip1}$. Our data confirm the results of Dellas et al (**17**) who demonstrated a feed back inhibitory loop between *cyclin E* and $p27^{kip1}$. This suggests the presence of a synergistic effect between both genes which will eventually lead to enhanced progression through the cell cycle as a consequence of the proliferative effect induced by increased *cyclin E* expression and the loss of the inhibitory function of $p27^{kip1}$.

Inactivation of *CDKIs* ($p27^{kip1}$, $p21^{waf}$, $p16^{INK4A}$) via reduced expression was reported in various human tumors (**18**). In HPV-associated cervical carcinoma, the situation is less clear since some studies showed that the tumor suppressor activity of these proteins is overcome through the action of the viral oncogenes E6/E7 without any change in their expression level (**18**), others showed that this applies to $p21^{waf}$ and $p27^{kip1}$ only whereas $p16^{INK4A}$ is usually down regulated (**18, 20**). Moreover, the HR-HPV types were shown to impair the function but not the expression of the $p21^{waf}$ and *p16*

^{INK4A} by rendering them insensitive to *cyclin-CDK* complex formation whereas $p27^{KIP1}$ is usually down-regulated (**20**).

In the present work, a stepwise decrease in $p27^{kip1}$ expression and a stepwise increase in $p16^{INK4A}$ were found in cervical epithelium as it progressed from normal to a neoplastic one. Our results regarding $p27^{kip1}$ expression are in agreement with previously published data (**6, 20, 21, 22**). In contrast, Shiozawa et al., (23) reported in their study a strong $p27^{kip1}$ expression in normal cervical epithelia which was markedly reduced to a negligible level in CIN samples. However, in ISCC cases they reported an increased expression of $p27^{kip1}$ and demonstrated that the *p27* protein was bound to cdk2 and cyclin E. Consequently, the authors concluded that, $p27^{kip1}$ expression may be involved in the growth regulation of NCT however aberrant function of the $p27^{kip1}$ may occur in ISCC of the cervix. Although we can not find a proper explanation for this complexity in the results, we assume that the mechanism proposed by Shiozawa et al. (23) might represent an alternative pathway for $p27^{kip1}$ inactivation in ISCC of the uterine cervix through binding and sequestration by *cdk2* and *cyclin E* which render it inactive just as the case with *p53* and *mdm2*.

In our study, $p16^{INK4A}$ overexpression was detected during the early stages of cervical carcinogenesis although no mutation was reported in any of the studied cases. We therefore assume that inactivation of $p16^{INK4A}$ gene in HPV-associated ISCC is possibly achieved via mechanism(s) other than gene mutations. Among which is the binding to- and sequestration by other cellular and/ or viral proteins. Our results are consistent with Volgareva et al. (**18**) and Tringler et al. (**24**) who reported $p16^{INK4A}$ overexpression in a high percentage of dysplastic and neoplastic lesions of the cervix uteri. They also mentioned that $p16^{INK4A}$ could be used as a surrogate marker for early diagnosis of cervical carcinoma. However, our results regarding $p16^{INK4A}$ gene mutations

in ISCC contrast with Tripathi et al. **(14)** who reported $p16^{INK4A}$ gene mutation in 15% of their ISCC cases. A difference in sampling methods, clinical and virological features of studied cases or a racial difference could be mentioned as possible explanations for the controversial results between the two studies.

An interesting finding in this study is the significant increase of $p21^{waf}$ which was recognized at CINIII and ISCC cases only. Although it is expected that increased expression of $p21^{waf}$ should suppress the progression of cells in the cell cycle and consequently suppress cell growth, all the cases that revealed $p21^{waf}$ overexpression had a high *Ki-67* positivity index. Several explanations could be mentioned in this regard including: 1) the interaction between HPV E7 oncoprotein and $p21^{waf}$ which abrogates its inhibitory effect on *cyclin/CDK4* activities**,** 2) the occurrence of checkpoint adaptation after constant stimulation, 3) altered or inhibited binding to the *CDK4/cyclin D1* complex, or 4) mutations in downstream targets of $p21^{waf}$ **(3, 6, 8,24)**.

In gynecologic oncology, valid prognostic factors are necessary to define biologically similar subgroups for analysis of therapeutic efficiency. In the present study *cyclin D1, p27* and a high *Ki-67* showed a prognostic relevance as increased expression of *cyclin D1 and Ki-67* as well as loss of *p27* were highly correlated with the standard clinicopathologic prognostic factors for ISCC. Our data regarding the prognostic value of *cyclin D1* are in agreement with Bae et al. (**16**) who demonstrated that *cyclin D1* overexpression is a poor prognostic factor in cervical carcinoma. Similarly, our data regarding *p27* are consistent with previous reports **(18)** which considered $p27^{KIP1}$ an independent prognostic factor that significantly correlates with poor survival in ISCC.

We conclude that, HPV-associated cervical carcinogenesis is a complex process including several pathways. According to our results, infection of cervical mucosa by HPV16 and 18 leads to deregulation of the cell cycle via altering the expression level of

certain genes. Whereas *p27* ^{KIP1}, *cyclin E, CDK4* and *p16*^{INK4A} aberrations are early events; *cyclin D1*and *p21*^{waf} aberrations occur late in cervical carcinogenesis. Together, these alterations lead to acceleration of the cell cycle with increased proliferation rate, as indicated by a high *Ki-67* index, and acquisition of more genetic damage. Our results also provide evidence that the application of immunohistochemical screening tests for *p16*^{INK4A}, *cyclin E* and *p27* ^{KIP1} could help in early diagnosis of cervical carcinoma especially in high risk groups, since alterations affecting the expression level of these proteins occur at an early stage of cervical carcinogenesis. Therefore they could be used as surrogate markers for early detection of ISCC and for monitoring patients with cervical dysplasia. On the other hand, *cyclin D1, p27* ^{KIP1}and *Ki-67* could be used as prognostic factors in cervical cancer.

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Table 1: Clinicopathologic features of invasive squamous cell carcinoma cases in relation to different studied markers.

Clinical features	*No.= 43*	*Cyclin D1 n=18*	*Cyclin E n=38*	*Cdk-4 n=40*	*P27 Klp1 n=37*	*P21 waf n=29*	*P16 n=40*	*Ki-67 n=36*
Tumor size								
<4cm	*15*	2	10	12	9	7	13	10
≤4cm	*28*	16	28	28	28	22	17	26
		(*p*=0.003)	**(*p*=0.003)**		**p=0.047**	**(*p*=0.001)**		**(*p*=0.008)**
Tumor type								
LK-SCC	13	7	9	13	7	10	2	8
LNK-SCC	26	10	25	24	26	17	24	24
SCC	4	1	4	3	4	2	4	4
					p=0.047			
Stromal invasion								
<1/2	15	6	13	15	12	9	14	12
≤1/2	28	12	25	25	25	20	26	24
					p=0.024	**p=0.037**		
Lymph nodes								
Positive	13	5	9	11	7	10	11	7
Negative	30	13	29	29	30	19	29	29
					p=0.001			p=0.001
Lympho-vascular								
Positive	7	5	5	5	4	5	5	6
Negative	36	13	33	35	33	24	35	30
					p=0.035			
Vaginal involvement								
Positive	12	6	10	10	7	8	10	11
Negative	31	12	28	30	30	21	30	25
						(*p*=0.037)		
FIGO Stage								
I&II	17	3	15	16	12	12	17	10
III&IV	26	15	23	24	25	17	23	26
		(*p*=0.008)			**p=0.002**			**p=0.019**

LNK-SCC: Large non-keratinized squamous cell carcinoma

LK-SCC: Large keratinized squamous cell carcinoma

SCC: Small cell carcinoma

Table 2: Summary of specific antisera and immunohistochemistry conditions

Protein	*Antibody*	*Dilution& incubation*	*Antigen pretreatment*	*Positive control*	*Stain*
Cyclin D1	P2D11F11 Novocastra	1:50	Microwave	Colon cancer	Nuclear
Cyclin E	13A3 Novocastra	1:80	Microwave	Breast cancer	Nuclear
CDK4	C-22 Santa Cruz Biotechnology	1:300	Microwave	Colon cancer	Nuclear
P21^{waf}	SX118, DAKO	1:50, 2h	Microwave	Epidermis	Nuclear
P27	SX53G8, DAKO	1:80, 1h	None	Breast cancer	Nuclear
P16	6H12 Novocastra Lab	1:40	Microwave	Epidermis	Nuclear
Ki-67	MIB 1, Dako	1:50	Microwave	Breast cancer	Nuclear

Table 3: The level of protein expression of different studied markers in normal, dysplastic and neoplastic cervical epithelium detected by immunohistochemistry

Marker	NCT (20)		CINI (n=18)		CINII (n=11)		CINIII (n=38)		ISCC (n=43)	
	Pos. Cases	PI*	Pos. cases	PI	Pos. cases	PI	Pos. cases	PI	Pos. cases	PI
Ki-67	5 (25%)	<0.1		6.5±0.5		10±0.45		21.6±9		40.5±11
Cyclin D1	0(0%)		0(0%)		0 (0%)		5(13.1%)	24.7±3.6	18(41.9%)	63.2±10.8
Cyclin E	0(0%)		3(16.7%)	18±1.1	4(36.4%)	30±4.4	21(55.3%)	60±10.2	38(88.4%)	97±13.8
CDK4	0(0%)		2(11.1%)	5±0.5	4(36.4%)	12±1.7	17(44.7%)	35±4.6	40(93%)	92±14.7
$p21^{waf1}$	3(15%)	2.8±0.2	1(5.5%)	7±0.5	1(9.1%)	9.2	16(42.1%)	58±7.1	29(67.4%)	89±10.6
$p27^{KIP1}$	20(100%)	90±8.6	18(100%)	77±10.5	7(63.6%)	60±11.7	10(26.3%)	20±5.5	6(13.9%)	9±1.1
$p16^{INK4A}$	0(0%)		0(0%)		4(36.4%)	24±4.7	24(63.1%)	70±12.6	40(93%)	98±12.0

PI= Positivity index

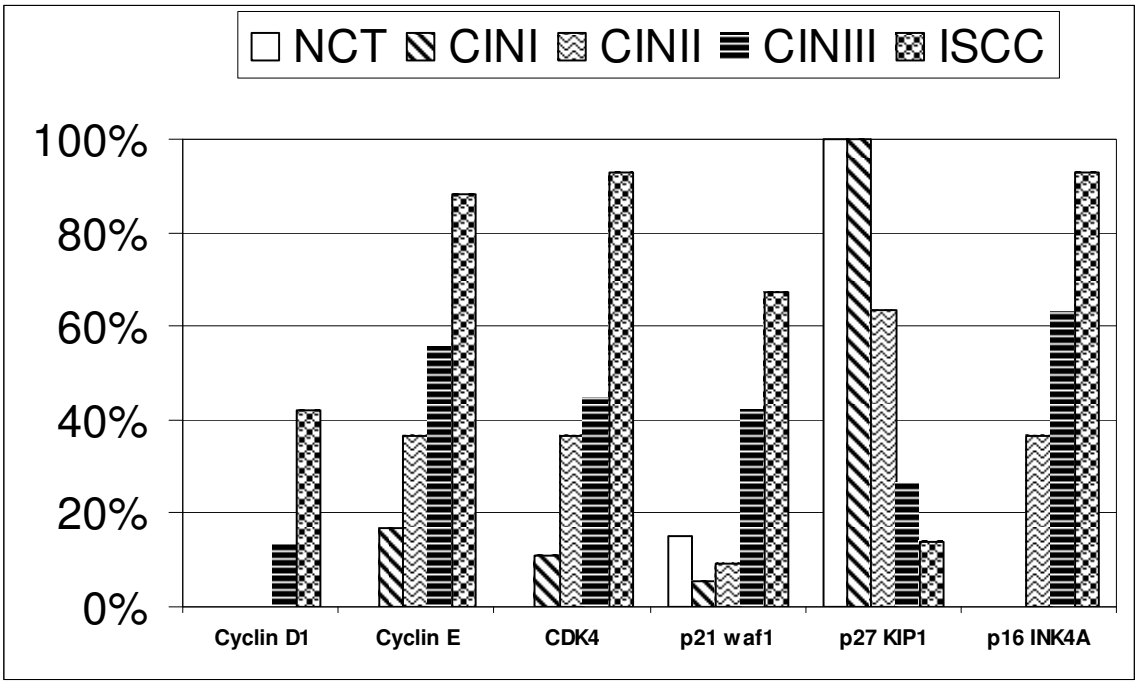
NCT
CINI
CINII
CINIII
ISCC
100%
80%
60%
40%
20%
0%
Cyclin D1
Cyclin E
CDK4
p21 waf1
p27 KIP1
p16 INK4A

Figure 4