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Full Length Research Paper

Relationship of HPVL1 and p16 expression with different cervical lesions

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p16 is a tumor suppressor protein and also a cyclin-dependent kinase inhibitor and HPV-L1 (L1) is a capsidic protein that is expressed in the early, productive phase of cervical carcinogenesis. To provide evidence for the evaluation of endometrial intraepithelial neoplasia (CIN), the expressions of p16 and $HPVL_1$ in different cervical diseases were determined and their relationship was explored. Immunohistochemistry was performed to detect the expressions of p16 and $HPVL_1$ in women positive for HPV among whom 38, 32, 28, 33 and 10 had CIN I, CIN II, CIN III, cervical squamous cell carcinoma (SCC) and cervical adenocarcinoma, respectively. Fourteen healthy subjects were recruited as controls. Results showed the positive rate of p16 in cervicitis, CIN I, CIN II, CIN III, cervical SCC and cervical adenocarcinoma patients was 0, 26.3, 81.2, 96.4, 100 and 90%, respectively, and that of $HPVL_1$ was 100, 65.8, 13.8, 0, 0 and 0%, respectively. p16(-)/$HPVL_1$(+) was frequently found in patients with cervicitis or cervical lesions of low grade and p16(+)/$HPVL_1$(-) was often noted in women with CIN III or cervical cancer. The cervical lesions of patients with p16(-)/HPVL1(-) or p16(+)/$HPVL_1$(+) might have no progression or undergo degeneration. Detection of p16 and $HPVL_1$ expressions plays an important role in the prediction of early cervical cancer and its progression.

Key words: Cervical tumor, cervical intraepithelial neoplasia, human papilloma virus, HPVL1 protein, p16.

INTRODUCTION

Cervical cancer is one of the most common malignancies in women. It is also very well known that human papilloma virus HPV undeniably plays a role in the development of most cervical cancers. However, only a minority of women positive for HPV develop cervical cancer. Therefore, some women will undergo unnecessary treatment, whereas conization will be delayed in others. The possibility of predicting the behavior of low-grade cervical lesions could therefore be of high value in clinical practice, potentially allowing an individualized management of cervical lesions depending on their progression risk. p16, a tumor suppressor protein and also a cyclin-dependent kinase inhibitor whose overexpression has repeatedly been reported to be typical of dysplastic and neoplastic epithelium of cervix (Klaes et al., 2001; Volgareva et al., 2004) slows down the cell cycle by inactivating the cyclin-dependent kinases that phosphorylate retinoblastoma protein (pRb) (Koh et al., 1995; Serrano et al., 1993). Many studies have proposed that p16 is a useful biomarker especially for HR (high risk)-HPV type-related cervical neoplasia and also for predicting squamous intraepithelial lesion (SIL) progression (Yildiz et al., 2007). Moreover, a few other studies have recently concluded that there exists a significant association between cervical lesion grade and p16 staining distribution and intensity (Lambert et al., 2006).

HPV-L1 (L1) is a capsidic protein that is expressed in the early, productive phase of cervical carcinogenesis and is progressively lost in the later proliferative phase when p16 gets overexpressed (Doorbar, 2005). As previous study shown, the L1/p16 expression patterns are related to the severity of cervical lesions and may serve as a valuable index for predicting prognosis and

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Table 1. p16 expression in the cervical diseases.

Group	n	p16(+)	P16(-)	Positive rate (%)
Cervicitis	14	0	14	0.0
CIN I	38	9	29	26.3
CIN II	32	26	6	81.2
CIN III	28	27	1	96.4
Cervical SCC	33	33	0	100.0
Cervical adenocarcinoma	10	9	1	90.0

determining a follow-up strategy for dysplastic lesions of the cervix (Negri et al., 2008). In the present study, the expressions of p16 and HPVL1 in cervicitis, cervical intraepithelial neoplasial I (CIN I), CIN II, CIN III, cervical squamous cell carcinoma (SCC) and cervical adenocarcinoma were determined and the relationship between p16 and HPVL1 was explored. Our study may provide evidence for the evaluation of endometrial intraepithelial neoplasia (CIN).

MATERIALS AND METHODS

Sample collection

Patients with CIN of different grades or cervical cancer were recruited from June 2009 to June 2010. Cervical tissues were collected for thinprep cytology test or post-operative pathological examination and the detection of HPV (HC2) was also performed. Then, HPV positive patients were then divided into the following groups: CIN I (n = 38), CIN II (n = 32), CIN III (n = 28), cervical squamous cell carcinoma (SCC) (n = 33) and cervical adenocarcinoma (n = 10). In addition, 14 healthy subjects were also recruited as controls. The mean age was 44 years (range: 22~63 years). All patients and controls had no history of radiotherapy and chemotherapy before study. This study has been approved by the Ethics Committee of People's Hospital of Henan Province.

Reagents

The cervical tissues were conventionally fixed, embedded and cut into consecutive sections (4 μm). Mouse anti-human p16 monoclonal antibody (Maixin Biotech, Co., Ltd) and HPVL1 kit (Advance, USA) were used in the present study. Detection of HPVL1 was carried out according to the manufacturer's instructions.

Determination of protein expressions

The p16 positive cells have brown nuclei and cytoplasm and L1 positive cells have brown nuclei. The grading of p16 expression was performed according to previously described by Negri et al. (2008): p16 positivity was defined as that the proportion of p16 positive cells in the 1/3 of subepithelial region with dysplasia is greater than 25%; HPVL1 positivity was defined as cells having HPVL1 expression in the nuclei.

Statistical analysis

Statistical analysis was performed with SPSS version 10.0 statistic software package. X^2 test was used for analysis. A value of $P<0.05$ was considered statistically significant.

RESULTS

Expression of p16 in the abnormal cervical cells

The p16 expression was found in the nuclei or cytoplasm. The positive rate of p16 was 0, 26.3, 81.2, 96.4, 100 and 90% in cervicitis, CIN I, CIN II, CIN III, cervical SCC and cervical adenocarcinoma patients, respectively showing significant difference among patients with cervicitis, CIN I and CIN II ($P<0.05$). No significant difference was noted among CIN III, cervical SCC and cervical adenocarcinoma patients (Table 1 and Figure 1).

L1 protein expression in the abnormal cervical cells

HPVL1 expression was mainly found in the nuclei. In cervicitis, CIN I and CIN II patients, the positive rate was 100, 65.8, 13.8, 0, 0 and 0%, respectively, showing significant difference. But HPVL1 expression was not observed in CIN III, cervical SCC and cervical adenocarcinoma patients (Table 2 and Figure 1).

Relationship between L1 and p16 expression

As shown in Table 3, L1(+)/P16(-) was frequently found in patients with cervicitis or CIN of low grade and P16(+)/HPVL1(-) observed in patients with CIN of high grade or cervical cancers. For patients who were P16(+)/HPVL1(+) or P16(-)/HPVL1(-), the cervical disease might have no progression or have the potential to degenerate.

DISCUSSION

Despite extensive studies of cervical cancer precursors, the interobserver variation in the histopathologic interpretation of cervical biopsy specimens still constitutes a dilemma (Stoler et al., 2001; Klaes et al., 2002). The search for a specific diagnostic biomarker to

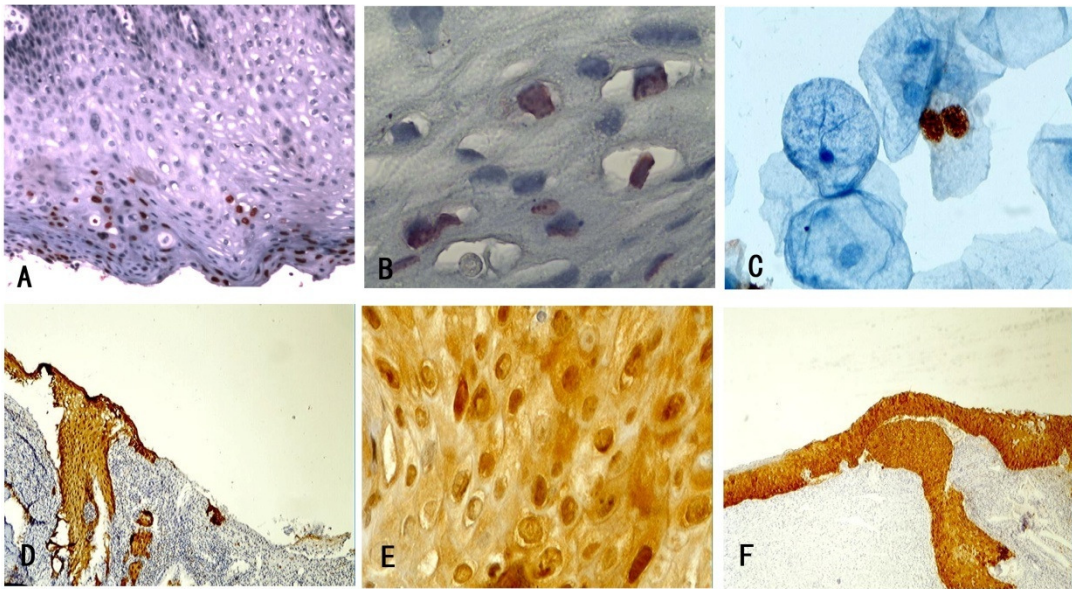

Figure 1. A) HPVL1 positive cells in CIN II patients; B) HPVL1 positive cells in CIN I patients; C) HPVL1 positive cells in cervicitis patients (TCT); D) p16 positive cells in CIN I patients; E) p16 positive cells in CIN II patients; and F) p16 positive cells in CIN III patients.

Table 2. HPVL1 expression in the cervical diseases.

Group	n	$HPVL_1(+)$	$HPVL_1(-)$	Positive rate (%)
Cervicitis	14	14	0	100.0
CIN I	38	25	13	65.8
CIN II	32	5	27	13.4
CIN III	28	0	28	0.0
Cervical SCC	33	0	33	0.0
Cervical Adenocarcinoma	10	0	10	0.0

Table 3. Expressions of p16 and HPVL1 in difference cervical diseases.

Group	n	$L_1(+)/P_{16}(-)$	$L_1(+)/P_{16}(+)$	$L_1(-)/P_{16}(+)$	$L_1(-)/P_{16}(-)$
Cervicitis	14	14(100.0%)	0	0	0
CIN I	38	23(60.5%)	2(5.3%)	7(18.4%)	6(15.8%)
CIN II	32	2(6.3%)	3(9.3%)	23(72.0%)	4(12.6%)
CIN III	28	0	0	27(96.4%)	1(3.6%)
Cervical SCC	33	0	0	33(100%)	0
Cervical adenocarcinoma	10	0	0	9(10.0%)	1(10%)

improve the interobserver reproducibility in the histologic diagnosis of cervical intraepithelial lesions revealed p16 plays an important role in the occurrence and development of cervical cancers and its precursors. Normally, p16 is a cell-cycle inhibitor that binds to cyclin-dependent kinase 4 (CDK4)/CDK6 and prevents the phosphorylation and subsequent inactivation of the retinoblastoma protein (pRB) (Kalof and Cooper, 2006). The down-regulation of p16 has been associated with carcinogenesis in a variety of organ systems (Leversha et al., 2003; Makitie et al., 2003). However, over-expression of p16 is frequently shown in cervical cancers and its precursors. Integration of HPV DNA into the host genome results in the over-expression of viral proteins E6 and E7 whose expression is associated with malignant transformation of the cervical epithelial cells (zur Hausen, 1994). These proteins can bind to and inactivate pRB and induces the production of p16 through a negative

feedback loop (Klaes et al., 2002). The pRb status has a remarkable influence on the expression of p16. The p16 expression in cervical lesions is hypothesized to be caused by the functional inactivation of pRb by HPV-E6 and E7 proteins. The p16 has been used as a good indicator of high-grade CIN (Kalof and Cooper, 2006). Immunocytochemistry for p16 has been performed in ThinPrep smears (Murphy et al., 2003) and cell block preparations and increasing studies support the finding that p16 is a useful marker for the diagnosis of CIN on cell blocks (Liu et al., 2007).

HPV L1 (L1) capsid protein is a nuclear protein expressed by all HPV subtypes during a productive infection. The production of this protein is linked to the maturation process of basal to superficial epithelial cells; therefore, and L1 is expressed strongly at the superficial layer of the epithelium (Ozbun and Meyers, 1998). L1 is expressed in the early productive phase of HPV infection and progressively is lost in the later transformation phase, when p16 becomes overexpressed (Doorbar, 2005). It has been demonstrated that L1 has prognostic relevance in mild-to-moderate dysplastic lesions, in which its expression indicates a greater tendency to regress compared with L1-negative lesions (Griesser et al., 2004; Rauber et al., 2008).

Clinical significance of HPVL1 in the cervical intraepithelial lesions

The L1 capsid protein is the major target for the cell-mediated immune response to HPV infections and normally is detectable during the productive stage of HPV disease. It is produced abundantly in mild-to-moderate dysplasia but rarely is observed in non-suspicious Pap smears or severe dysplasia and it is not produced in carcinomas (Rauber et al., 2008; Melsheimer et al., 2003; Birner et al., 2001). The L1 capsid protein reportedly was positive in 30% of low-grade squamous intraepithelial lesion (LSIL), 12% of high-grade squamous intraepithelial lesion (HSIL) and 0% of SCCs in liquid-based cytology (Ostor, 1993) and in 43.7% of LSILs and 33.3% HSILs in cervical smears (Melsheimer et al., 2003). In the study of Xiao et al. (2010) L1 capsid protein was positive in 69.79% of cervicitis, 83.53 % of CIN I, 41.81% of CIN II, 3.13% of CIN III and 0% of SCC. Cytologic diagnosis revealed a higher expression rate in LSIL than in a typical squamous cells of undetermined significance (ASCUS) and HSIL + SCC. In 71 ASCUS/LSIL without treatment, no L1-positive cases progressed in cytology; 18.75% of L1-negative cases progressed to ASC-H/HSIL (Xiao et al., 2010). In addition, the positive rates of HPVL1 decreased gradually according to the severity of cervical neoplasia (Yu et al., 2010).

Our results in the present study showed the HPVL1 expression reduced with the increase of the grade of cervical lesions, and was almost not found in CIN III and cervical cancers which is consistent with previously reported in Griesser et al. (2004). This finding indirectly suggests the deficiency of L1 expression predicts the increased risk of malignant transformation of cervical diseases.

Clinical significance of p16 in the cervical intraepithelial lesions

Study shows in biopsies of CIN I, p16 is diffusely expressed in about 60% of cases and is typically associated with high-risk human papillomavirus infection (Klaes et al., 2001). In the study of Yildiz et al. (2007), all HSIL cases and 80% of LSIL cases were positive for p16. In a meta-analysis, among normal smears, only 12% were positive for the biomarker compared to 45% of ASCUS and LSIL and 89% of HSIL smears. Similarly, in histology only 2% of normal biopsies and 38% of CIN I showed diffuse staining for p16INK4a compared to 68% of CIN II and 82% of CIN III (Tsoumpou et al., 2009). In addition, the immunointensity and cells that were positive for p16 were enhanced according to increased pathologic grade and differed statistically between CIN-I and CIN-II/CIN-III as well as SCC (Yu et al., 2010). Our results revealed the p16 expression increased with the increase of the grade of cervical lesions. In CIN III or cervical cancer patients, p16 was over-expressed which suggests high expression of p16 is an early event in the progression from normal cervical tissues, CIN to cervical cancer.

Role of detection of p16 and HPVL1 expressions in the diagnosis of cervical diseases

In recent years, a few studies reported the detection of both p16 and HPVL1 expressions plays an important role in the prediction of cervical disease progression. The study of Negri et al. (2008) showed that the combination of both L1 and p16 may be useful in the estimation of the biologic risk of low-grade squamous lesions of the cervix uteri. Compared with p16 alone, the combination with L1 may be particularly useful in assessing those lesions that are still in the productive phase of carcinogenesis. Yu et al. (2010) also noted HPVL1/p16 expression patterns were related to the severity of cervical lesions. Yoshida et al. (2008) performed liquid-based cytology in 63 patients and found that the detection rate of P16(-)/L1(+) in LSIL, HSIL and SCC was 44, 0 and 0%, respectively, and that of P16(+)/L1(-) was 82, 88 and 100%, respectively. This finding suggests the detection of both L1 and p16 can be used to predict the progression of cervical disease. Our results indicated that P16(-)/HPVL1(+) was frequently noted in patients with cervicitis or CIN of low grade, and P16(+)/HPVL1(-) often found in CIN III or cervical cancer patients. Furthermore, P16(+)/HPVL1(+) or P16(-)/HPVL 1(-) cervical diseases may have no progression or have

the potential to resolve. P16(+)/HPVL1(-) patients had the high risk for the development into CIN of high grade or even cervical cancer and should be followed up closely. Currently, in the screening of cervical cancer, cytology has poor sensitivity and HPV positivity has low prediction rate of CIN of high grade. Thus, both techniques may lead to over-treatment for CIN or misdiagnosis of cervical cancers at early stage.

Detection of both p16 and HPVL1 can predict the progression of cervical diseases and provide evidence for the monitoring of patients with high risk for cervical cancers and there is no difference in the age among patients with different cervical lesions. Therefore, our results are beneficial for the timely treatment of cervical cancer at the early stage and avoidance of over-treatment among patients having the potential to resolve.

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