Androgen replacement therapy contributes to improving lower urinary tract symptoms in patients with hypogonadism and benign prostate hypertrophy: A randomised controlled study

Etiological role of human papillomavirus infection for inverted papilloma of the bladder

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ABSTRACT

The status of human papillomavirus (HPV) infection in urothelial inverted papilloma was

examined in the present study. Formalin-fixed and paraffin-embedded tissues from eight cases

of inverted papilloma of the bladder were studied. The presence of HPV-DNA was examined by

modified GP5/6+PCR using archival tissue sections by microdissection. HPV genotype was

determined with a Hybri-Max HPV genotyping kit. Immunohistochemical analysis for

p16-INK4a, mcm7, HPV-E4, and L1, and *in situ* hybridization (ISH) for the HPV genome were

performed. HPV was detected in 7 of 8 cases (87.5%) of inverted papilloma. Three cases were

diagnosed as inverted papilloma with atypia, while the remaining 5 were typical cases. HPV-18

was detected in two cases, including one inverted papilloma with atypia, and HPV-16 was

detected in four cases, including one inverted papilloma with atypia. Multiple HPV type

infection was detected in one typical case and one atypical case. High-risk HPV was present in

all HPV-positive cases. Cellular proteins, p16-INK4a and mcm7, which are surrogate markers

for HPV-E7 expression, were detected in all HPV-positive cases, and their levels were higher in

inverted papilloma with atypia than in typical cases. In contrast, HPV-E4 and L1, which are

markers for HPV propagation, were observed in some parts of the typical inverted papilloma

tissue. High-risk HPV infection may be one of the causes of urothelial inverted papilloma, and

inverted papilloma with atypia may have malignant potential.

Key Words: human papillomavirus, inverted papilloma, *in situ* hybridization, p16, mcm7

INTRODUCTION

Certain types of human papillomavirus (HPV), which are transmitted through sexual

contact, are thought to be causative agents of cervical cancer and some other cancers. HPV is

highly prevalent in sexually active young men and women. However, many studies have

suggested that men only act as a reservoir for HPV transmission, as urothelial malignancies are

considered to be induced rarely by HPV. However, HPV has been detected in some cases of

urothelial carcinoma in the bladder [Barghi et al., 2005; Moonen et al., 2007]. Bladder cancer

generally develops in men over 60 years old in contrast to cervical cancer in younger women,

suggesting that HPV is unlikely to induce bladder cancer with a high incidence rate. Indeed,

HPV-DNA detection is much less frequent in bladder cancer than in cervical cancer, and

various rates of positivity for HPV-DNA in bladder cancer have been reported [Barghi et al.,

2005; Moonen et al., 2007; Tenti et al., 1996; Kamel et al., 1995]. Thus, the etiological role of

HPV in bladder cancer is still controversial.

Inverted papilloma is a rare benign urothelial tumor that develops predominantly in the

bladder, and accounts for less than 1% of all urothelial neoplasms [Sung et al., 2006b]. This

type of tumor is characterized pathologically by inverted growth of transitional epithelia with no

atypia. Generally, inverted papilloma occurs more frequently in men than in women, and the

most common target age is 30 to 60 years old, which is younger than bladder cancer [Sung et al.,

2006b]. Although the etiological factors associated with inverted papilloma have not been

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established, HPV-DNA has been detected in some cases of inverted papilloma [Chan et al.,

1997]. A recent study reported a high prevalence (35%) of HPV infection in the urinary tracts

(urethra and urine) of 142 men with urethritis [Shigehara et al., 2010], and morphological

abnormal cells suggestive of high-risk HPV infection were identified on Papanicolaou test of

scraped cells from the lower segment of the urethra, suggesting that persistent HPV infection in

the urinary tract may be a cause of bladder neoplasm.

Here, in the present study, the presence of HPV-DNA in the tissue of inverted

papilloma of the bladder was examined using a microdissection procedure, and the status of

HPV infection in the tissue was also investigated using an immunohistochemical (IHC) method

with detection of HPV-related proteins and by *in situ* hybridization (ISH) analysis for

HPV-DNA.

MATERIALS AND METHODS

Patients

Eight cases of inverted papilloma of the bladder treated at Kanazawa University

Hospital between 2000 and 2009 were investigated in this study. Histopathological diagnosis of

inverted papilloma was made based on 2004 World Health Organization classification of

noninvasive urothelial tumors [Lopez-Beltran et al., 2004]. Written informed consent for usage

of these samples had already been obtained from all patients before surgical resection, in

accordance with the protocol approved by the Ethics Committee of Kanazawa University

Graduate School of Medicine.

Samples

The inverted papilloma tissues were fixed immediately in 20% formalin, embedded in

paraffin, and stored at room temperature. The cases were classified pathologically into two

groups: the first was composed of normal urothelial cells (classical or typical inverted

papilloma), showing thin anastomosing trabeculae of urothelium that grow downward into the

stroma and absence of a papillary component; and the second was inverted papilloma with

atypia (atypical inverted papilloma), which had foci of atypia in a background of classic

inverted papilloma, as reported previously [Broussard et al., 2004]. Seven paraffin-embedded

normal mucosal samples, which were obtained by random bladder biopsy from another seven

patients with inflammatory bladder disorder, were used as negative controls.

HPV-DNA analysis and typing

DNA was extracted from paraffin-embedded inverted papilloma tissue and controls by

microdissection using the Pinpoint Slide DNA Isolation System™ (Zymo Research, Orange,

CA). The extracted DNA was stored at –30°C until use.

DNA quality was confirmed by amplifying the β-globin gene as an internal control by PCR. HPV-DNA was amplified using a modified GP5+/GP6+ PCR method as reported previously [Yamada et al., 2008]. HPV was defined as positive when a band of about 140 bp was observed after electrophoresis of 8 μL of PCR product on 2.0% agarose gels and staining with ethidium bromide.

HPV genotyping was performed using an HPV GenoArray Test Kit (HybriBio Ltd., Chaozhou, China). This assay can determine 21 HPV types, including 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), 5 low-risk HPV types (6, 11, 42, 43, and 44), and two unknown-risk types (53 and CP8304), by the flow-through hybridization technique using HPV-DNA amplified by PCR [Shigehara et al., 2010].

In situ **hybridization for HPV-DNA**

ISH was performed to detect HPV-DNA in tumor tissue using an HPV-detection kit according to the manufacturer's instructions (Dako GenoPoint System K0620; Dako, Carpinteria, CA). A wide-spectrum probe (Y1404; Dako) for 13 high-risk HPV-DNA (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), and low-risk HPV-DNA (HPV types 6, 11) probe (Y1411; Dako), and HPV types 16, 18 probe (Y1412; Dako) were hybridized with denatured DNA on tissue samples. HPV-DNA signals were visualized as brownish staining.

Hematoxylin was used to counterstain the cell nuclei in all specimens.

Immunohistochemistry for HPV-related proteins

The procedure for IHC staining was performed using a Dako ChemMate ENVISION

Kit/HRP(DAB)-universal kit (K5007) according to the manufacturer's protocol (Dako). Tissue

specimens were stained with mouse monoclonal antibodies against p16-INK4a

(Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) and against mini chromosome

maintenance protein-7 (mcm-7) (Abnova, Taipei, Taiwan), and against HPV-L1 protein

(Viroactiv; Virofem Diagnostica GmbH, Mainz, Germany). HPV-E4 staining was performed

using a type-specific rabbit polyclonal antibody against E4 proteins of HPV-6, -11, -16, -18, and

-33 according to the protocol described previously [Doorbar, 2006].

Antigen retrieval was performed by heating tissue sections at 95°C in Retrieval Solution,

pH 9 (S2367; Dako) for 40 min. After blocking endogenous peroxidase activity using 3%

hydrogen peroxide for 5 min, the sections were incubated with the primary antibodies at room

temperature for 30 min, followed by incubation for 30 min with peroxidase-labeled secondary

antibody (a mixture of rabbit and mouse antibodies) combined with dextran-polymers.

Brownish staining for target proteins on tissue slides was developed using DAB, and

counterstained with hematoxylin. The expression of each protein was scored according to the

following scale: Distribution of signals were defined as 0 (<5% of the cells were positive), 1+

(some: 5%–30% of the cells were positive), 2+ (many: 30%–50% of the cells were positive) and

3+ (extensive: $\geqq$50% of the cells were stained), The intensity of the signals was as 0 (not

stained), 1+ (moderately stained) and 2+ (strongly stained). We calculated the score as

multiplying the distribution score and the intensity score, and the expression levels of p16 and

mcm7 in typical and atypical inverted papilloma were compared.

RESULTS

Detection of HPV genomes in inverted papilloma tissue

Eight cases of inverted papilloma were investigated, and all results are summarized in

Table I. Five cases (case 3, 4, 5, 7, 8) were diagnosed as typical inverted papilloma

characterized by anastomosing cords and thin nests of normal urothelium growing down from

the surface of the epithelium, and often formed glandular or gland-like structures (Fig. 1A, B).

In contrast, three cases (case 1, 2, 6) were composed of various degrees of abnormal cell nests

containing cells of increased chromatin, irregular nuclear contours, and increased

nuclear/cytoplasmic ratios, with a loss of cell polarity (Figs. 1C, D).

HPV-DNA was detected in seven cases (87.5%). HPV-16 was identified in four cases,

including three typical and one atypical inverted papilloma. HPV-18 was detected in one typical

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and one atypical inverted papilloma. Multiple HPV type infection was observed in two cases

(one typical and one atypical inverted papilloma). The former was positive for HPV types 6, 16,

and 33, and the latter was positive for HPV types 33, 58, and 68. High-risk HPV types were

identified in all of HPV-positive cases.

ISH analysis revealed the presence of high-risk HPV-DNA in the nuclei of most of the

tumor cells in all HPV-positive cases (Fig. 2A). Different signal patterns were observed

between typical and atypical inverted papilloma. Punctate signals, which may represent

HPV-DNA integration, were predominant in atypical inverted papillomas (Fig. 2B, Table I),

whereas diffuse and intense signals, which are due to plasmid state of HPV-DNA, were seen in

typical inverted papilloma (Figs. 2C, Table I). HPV-DNA signals for HPV-16 and HPV-18

were detected in cases 1, 3, 4, 5, 6, and 7. On the other hand, no HPV-DNA signals were

detected in one HPV-negative case. HPV-6 and HPV-11 signals were seen only in case 3

infected with HPV-6 (Fig. 2D, Table I). The HPV type determined with ISH was consistent

with that indicated by the HPV genotyping test.

Both PCR method and ISH analysis demonstrated that HPV-DNA was not detected in

any control bladder specimens.

Expression of cellular proteins p16 and mcm7, which are surrogate markers of high-risk

HPV-E7 protein, in inverted papilloma

Cellular protein p16 is an inhibitor of cyclin-dependent kinase that stops the cell cycle.

The expression can be enhanced by high-risk HPV-E7 protein, and the cell cycle inhibitory

function of p16 is overridden in E7-expressing cells [Shai et al., 2007; Klaes et al., 2001].

Expression of mcm7 is also induced by E7 and contributes to cellular DNA replication [Brake et

al., 2003]. These cellular proteins are therefore regarded as surrogate markers for the expression

of E7 protein of high-risk HPV type in uterine cervical intraepithelial neoplasia (CIN) and

cervical cancer. Both p16 and mcm7 were expressed in all HPV-positive cells in both typical

and atypical inverted papilloma (Figs. 3A, B). The p16 and mcm7 signals were observed

predominantly in the nucleus, and mcm7 was expressed faintly in the cytoplasm in some cells.

The p16-positive inverted papilloma cells were observed more frequently than those positive for

mcm7. Neither p16 nor mcm7 signal was observed in one case, which was negative for

HPV-DNA, or in seven control specimens (Figs. 3C, D).

The mean expression levels of these proteins were compared using a scoring system

that recorded the distribution of the signals. Significant higher scores for mcm7 and marginally

higher scores for p16 were observed in atypical than in typical inverted papilloma (Fig. 4). The

levels of p16 or mcm7 expression represent proliferative activity induced by HPV infection,

suggesting that atypical inverted papilloma may be more aggressive than typical cases.

HPV infection status in inverted papilloma

There are two types of HPV infection, *i.e.*, reproductive and abortive infection. The

HPV-E4 protein is able to recognize the keratin network in host cells and stop cell division,

which may facilitate the release HPV particles [Doorbar, 2006]. This protein is expressed

abundantly during reproductive infection. HPV-E4 protein for each HPV type, such as HPV-6,

-16, -18, and -33, was detected in all HPV-positive inverted papillomas (Table I, Figs. 5A, B, C),

while no signals were seen for HPV-11-E4 protein (Table I, Fig. 5D). The E4 antibody was

HPV type specific. HPV-E4 was expressed more strongly in the cytoplasm of typical inverted

papilloma (Fig. 5C) than in atypical inverted papilloma (Fig. 5A). Curiously, nuclear staining

was observed occasionally in some cases (Fig. 5B). HPV-E4 protein was not expressed in one

HPV-negative inverted papilloma or in any controls.

HPV-L1 protein is the major capsid protein that assembles to form the viral shell. L1

protein was expressed widely only in case 4 (Fig. 6A), while a low level of expression was seen

in limited parts of the inverted papilloma tissue of cases 1, 2, 3, and 6. The expression was

limited to the apical sites of glandular structures and superficial layers of transitional cell

epithelium in inverted papilloma (Figs. 6B, C). L1 protein expression was not observed in one

HPV-18-positive case (case 5) and one HPV-16-positive case (case 7), although these were

typical inverted papilloma cases. Furthermore, this protein was not detected in atypical sites of

atypical inverted papilloma, one HPV-negative inverted papilloma, or control urothelial cells

(Fig. 6D).

DISCUSSION

Potts et al. [1963] first reported a benign bladder tumor designated as inverted

papilloma in 1963. Traditionally, inverted papilloma has been regarded as a benign neoplasm or

a hyperplastic reactive lesion. However, cases with recurrence or those with synchronous or

metachronous urothelial carcinoma have generated uncertainly with regard to its malignant

potential. A systematic review of 322 cases of inverted papilloma in the bladder indicated that

the recurrence rates was 4% and the incidences of associated synchronous urothelial carcinoma

and development of subsequent urothelial carcinoma were 6% and 3%, respectively [Cheng et

al., 2005]. Overaccumulation of p53, overexpression of HER-2/neu, and increased proliferation

index have been identified in some inverted papillomas, suggesting that it may be susceptible to

malignant transformation [Urakami et al., 1996; Cheon et al., 1995]. However, the association

of inverted papilloma with such molecular events is less frequent than in bladder urothelial

carcinoma [Sung et al., 2006a]. Some studies have doubted the malignant potential of inverted

papilloma, as its recurrence is very rare and inverted papilloma with atypia does not show high

proliferation activity [Sung et al. 2006a; Broussard et al., 2004].

The etiology of inverted papilloma has not been determined. One study suggested an

association between HPV infection and inverted papilloma of the bladder [Chan et al., 1997]. In

this previous study, in which the presence of the HPV genome was examined by HPV

type-specific PCR for HPV types 6, 11, 16, 18, 31, and 33, HPV was detected in 60% (6/10) of

cases of inverted papilloma. HPV-16 and HPV-18 were detected in 1 and 6 cases, respectively.

The results of the present study indicated a strong association between high-risk HPV infection

and inverted papilloma, as high-risk HPV-DNA was identified in seven (87.5%) of eight cases

of inverted papilloma. Furthermore, the localization of HPV-DNA was confirmed by ISH

analysis using specific probes for HPV-16, -18, and high-risk HPV types. HPV-16 was the most

common type. Low-risk HPV *i.e.*, HPV-6 was found in one case of inverted papilloma, but this

case showed co-infection with two high-risk HPV types. These findings suggested that high-risk

HPV is likely to be one of the causes for the development of inverted papilloma. A recent study

indicated a high prevalence rate (35%) of high-risk HPV infection in the lower urinary tract

(urethra and urine) in 142 male patients with urethritis, suggesting that incidental HPV infection

occurs not only in the external genitalia but also in the lower urinary tract [Shigehara et al.,

2010]. It is not surprising that HPV could infect urothelium in the urinary bladder and consistent

HPV infection may result in the development of urothelial neoplasm.

Pathologically, two different entities were observed among the present cases of

inverted papilloma. Some cases had foci of atypical urothelium composed of atypical cells with

nuclear enlargement, increased chromatin, and thickening of the nuclear membrane, in a

background of the classic inverted papilloma. Such cases were reported as inverted papilloma

with atypia [Broussard et al., 2004]. On the other hand, typical inverted papilloma was

composed of anastomosing cords and thin nests of normal urothelium growing down from the

surface epithelium, and sometimes accompanied with gland-like or glandular structures. In the

present ISH analysis of HPV-DNA, diffuse (cases 3, 4, 7), punctate (cases 1, 2, 6), and mixed

diffuse and punctate staining patterns (cases 5) were observed in the nuclei of inverted

papilloma cells. It has been reported that the diffuse patterns represent the episomal state of the

HPV genome in host cells, while punctate patterns indicate integration of the HPV genome into

the host cells [De Marchi Triglia et al., 2009]. Integration is observed frequently in high-grade

CIN and cervical cancer [De Marchi Triglia et al., 2009; Kalof et al., 2005], while the episomal

state is seen in papilloma or in lower grade CIN [Cooper et al., 1991]. Interestingly, punctate

signals (integration) were observed predominantly in atypical inverted papilloma, while diffuse

signals (episomal) were seen in typical cases.

The loss of p16 expression is often a critical event in the progression of tumors in

many organs, including bladder cancer [Rocco et al., 2001]. In fact, some studies have indicated

that p16 protein is downregulated in 19% to 60% of bladder cancers [Krüger et al., 2005; Yang

et al., 2002; Niehans et al., 1999]. In contrast, it is well known that upregulation of p16 in the

presence of HPV-E7 protein plays an important role in the process of carcinogenesis in cervical

cancer [Shai et al., 2007; Klaes et al., 2001] and head and neck cancer [Hafkamp et al., 2003].

The mcm7 gene is also responsive to E2F, and is used as another surrogate marker expressed in

high-grade CIN and cervical cancer [Brake et al., 2003]. Therefore, we also investigated the expression of p16 and mcm7 proteins, which are surrogate markers of E7 protein. Higher levels expression of p16 and mcm7 indicate higher activity of cell division with high-level E7 protein expression. In the present study, p16 and mcm7 were expressed in all HPV-positive cells in both typical and atypical inverted papilloma, supporting the suggestion that HPV infection may be associated with the development of inverted papilloma of the bladder. Furthermore, p16 and mcm7 were expressed at higher levels in atypical than in typical inverted papilloma. These observations suggest that cell growth is more aggressive for atypical inverted papilloma. The findings of HPV-DNA integration and high-level expression of p16 and mcm7 proteins in atypical inverted papilloma suggested its potential for malignant conversion. The present findings suggest that high-risk HPV may be a causative agent of urothelial inverted papilloma, and atypical inverted papilloma may be a precursor of urothelial cancer. On the other hand, Broussard et al. excluded the potential of atypical inverted papilloma for malignant progression in experiments regarding Ki-67 and cytokeratin-20 expression in atypical inverted papilloma tissue [Broussard et al., 2004]. It is not surprising that expression levels of these cell proliferation markers are reduced in atypical inverted papilloma in comparison with carcinoma, as atypical inverted papilloma is thought only to be a precursor lesion for cancer.

The main limitation of this study was the small number of specimens. Therefore, it will be important to confirm our hypothesis by further studies with larger numbers of specimens.

Further studies are also needed to determine whether expression of p16 and mcm7 coincides

with HPV infection in randomized samples of bladder cancer to evaluate the possible etiological

role of HPV in urothelial carcinoma.

If HPV is one of the causative factors of inverted papilloma, this would raise the

question of whether inverted papilloma is a source of HPV propagation or of abortive infection.

In women, HPV infection is thought be reproductive in condyloma acuminate and CIN 1.

However, it changes to an abortive infection in high-grade CIN and cervical cancer after many

years of persistent infection. We also investigated the expression of HPV-E4 and L1 proteins

that are concordant with cell cycle arrest and HPV propagation [Doorbar, 2006]. E4 protein

expression was observed in all cases of HPV-positive inverted papilloma. Although E4

expression is often observed in the cytoplasm of differentiating epithelial cells (Figure 5A, C),

in some situations the expression was apparent in the nucleus of atypical inverted papilloma

(Figure 5B). This may represent a different form of the protein, and it is interesting that

N-terminal cleavage of HPV-16 E4, which removes its keratin binding motif, can lead to

nuclear accumulation of E4 in experimental systems [Roberts et al., 1997; Wang et al., 2004].

We do not know the exact reason or mechanism for such aberrant expression patterns for E4 in

atypical inverted papilloma. On the other hand, L1 protein expression was observed in five

cases of inverted papilloma, although it was restricted to typical sites of glandular structures and

superficial layers of transitional cells. The signals were not detected in two HPV-positive cases

that were typical inverted papilloma. These findings suggested that HPV propagation may occur

in limited parts of the inverted papilloma tissue, but its extent may be limited.

In conclusion, high-risk HPV may be one of the etiological factors for inverted

papilloma and inverted papilloma with atypia may have malignant potential.

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FIGURE LEGENDS

Fig. 1. Histological findings of inverted papilloma stained with hematoxylin & eosin (HE). (A) Gland-like structure in typical inverted papilloma of case 3 (HE staining, magnification ×100) (B) Typical inverted papilloma of case 3, (HE staining, magnification ×400) (C) Inverted papilloma with atypia of case 1 (HE staining, magnification ×100). (D) Inverted papilloma with atypia of case 2 (HE staining, magnification ×400).

Fig. 2. Localization and signal patterns of HPV-DNA in the inverted papilloma (inverted papilloma) by *in situ* hybridization analysis. (A) High-risk HPV-DNA signals in typical inverted papilloma of case 3 (magnification ×100) (B) Punctate signals of high-risk HPV-DNA in inverted papilloma with atypia of case 2 (magnification ×400). (C) Diffuse and intense signals of HPV-16/18-DNA in typical inverted papilloma of case 6 (magnification ×400). (D) No signals of HPV type 6/11-DNA in inverted papilloma of case 5 (magnification ×40).

Fig. 3. Expression patterns of p16 and mcm7 proteins in the inverted papilloma (inverted papilloma) (magnification ×400). (A) The p16 signals in the nuclei of inverted papilloma cells of case 1. (B) The mcm7 signals in the nucleus of inverted papilloma cells of case 1. (C) No signals of mcm7 in control transitional cells. (D) No signals of p16 in control transitional cells.

Fig. 4. Comparison of expression levels of p16 and mcm7 proteins in inverted papilloma with atypia and typical inverted papilloma (in seven HPV-positive cases) (Mann-Whitney *U*-test; *P* < 0.05).

Fig. 5. Expression patterns of HPV-E4 protein in inverted papilloma (inverted papilloma) (magnification ×400). (A) Abundant expression of HPV-18-E4 in the cytoplasm of inverted papilloma with atypia of case 1. (B) HPV-16-E4 expression in the nuclei of inverted papilloma

of case 3. (C) Expression of HPV-6-E4 protein in the cytoplasm of apical parts of gland-like structures in inverted papilloma of case 3. (D) No signals for HPV-11-E4 in inverted papilloma of case 1 (negative control).

Fig. 6 Expression patterns of high-risk HPV-L1 protein in the inverted papilloma (inverted papilloma) (magnification ×400). (A) Abundant cytoplasmic expression of L1 in inverted papilloma of case 1. (B) L1 expression in the apical parts of gland-like structures of inverted papilloma of cases 2. (C) L1 expression in the superficial layers of transitional cell epithelium within inverted papilloma of case 4. (D) This protein was not expressed in atypical parts of inverted papilloma with atypia (case 1).

Table1. Demographic data of inverted papilloma cases

	case 1	case 2	case 3	case 4	case 5	case 6	case7	cases8
Age	36	58	35	49	78	37	66	53
Sex	Male	Male	Female	Male	Male	Male	Male	Male
Pathological finding	atypical	atypical	typical	typical	typical	atypical	typical	typical
β globin	+	+	+	+	+	+	+	+
HPV type	18	33,58,68	6,16,33	16	18	16	16	negative
In situ hybridization								
high-risk HPV probe	2+ (punctate)	2+ (punctate)	1+ (diffuse)	1+ (diffuse)	1+ (diffuse/ punctate)	1+ (punctate)	2+ (diffuse)	−
type 6/11 probe	−	−	2+ (diffuse)	−	−	−	−	−
type 16/18 probe	2+ (punctate)	−	+/− (diffuse)	2+ (diffuse)	2+ (diffuse/ punctate)	2+ (punctate)	2+ (diffuse)	−
Immunohistochemistory (area/intensity/location)								
p16	3+/3+/N	3+/2+/N	2+/2+/N	2+/3+/N	2+/2+/N	3+/2+/N	2+/1+/NC	−/−/−
mcm7	2+/3+/NC	2+/2+/NC	1+/2+/NC	2+/2+/NC	1+/1+/NC	2+/3+/NC	1+/2+/NC	−/−/−
HPV-E4 type11(contro	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
type6	*	*	3+/2+/C	*	*	*	*	*
type16	*	*	2+/2+/NC	2+/1+/C	*	3+/2+/N	1+/1+/NC	*
type18	+/+/NC	*	*	*	3+/1+/C	*	*	*
type33	*	2+/2+/C	2+/1+/N	*	*	*	*	*
HPV L1	+/+/C	+/+/C	+/+/C	2+/1+/C	−/−/−	+/+/C	−/−/−	−/−/−

Immunohistochemical; signal area; −;<5%, 1+; <5-30%, 2+; 30-50%, 3+; ≧50%

signal intensity; −; negative 1+; moderate, 2+; strong

signal location; N; nucleus, C; cytoplasm, NC; nucleus and cytoplasm

*; not examined