Abnormal "low grade" transformation zone: current diagnostic gold standard

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Summary

The aim of this work was to examine different methods of investigation in the diagnosis of the abnormal "low grade" transformation zone of the portio.

Over a period of one year 41 patients subjected to colposcopic examination underwent exo-endocervical sampling for oncologic evaluation and for detection of viral and bacterial infections (HPV, HSV, adenovirus, mycoplasmas and chlamydia trachomatis), as well as portio biopsy.

A 65.8% correlation was found between cytology and the HPV-DNA test results, while histology and the presence of the HPV virus agreed in 51.4% of cases. In those cases in which minimal histological alterations were found (koilocytosis) a high percentage of HPV negativity was found. In discordant negative cytologic tests that were however positive for HPV by PCR, the genotypes identified were always 6 and 11.

Key words: Cervix; Colposcopy; Cytology; Histology; Genital infections; Mycoplasma; HPV; HSV; Adenovirus; Chlamydia trachomatis.

Introduction

The characterization of a low-grade cervical lesion is the resultant of a combination of instrumental examinations that, if used singly, all display several limitations as regards the diagnostic evaluation: colposcopy, cytologic testing, histologic examination and testing for the presence of pathogenic organisms.

Colposcopy fails to distinguish lesions with a high risk of progression from those with a low risk [1].

Likewise, cytologic examination, used in screening for preneoplastic and neoplastic lesions of the uterine cervix, displays low sensitivity in the diagnosis of HPV infections [2].

Histological examination is considered "the gold standard" in assessing the abnormal transformation zone of the uterine cervix. Pathological examination does not always confirm the suspected diagnosis [3].

HPV infection can lead to benign histological alterations: papillomatosis, acanthosis, hyperplasia of the parabasal cells, keratinization, nuclear degeneration (chromatin condensation and margination), vacuolization of the intermediate layer cells and binucleated cells. Histological alterations that are instead an indication of atypia include: "atypical" koilocytosis, the presence of cells with multiple giant nuclei, hypercellularity, alteration of the chromatin pattern and the shape of the nucleus, and abnormal mitosis [4].

HPV infection diagnosis is generally cytologic, colposcopic and histologic.

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The three diagnostic methods (cytology-colposcopy-histology) do not always agree in the diagnosis of the various forms of virus infection and are not able to identify the various different types of HPV present in the lesion [5].

Over the last ten years molecular biology techniques have been used to obtain precious information concerning the epidemiology and pathogenesis of Papillomavirus infection. They enable a differential diagnosis to be made in the case of non-pathognomonic lesions, provide information concerning the type of HPV responsible for the lesion, and help the clinician to assess the prognosis and choose the most suitable treatment. On the other hand, these techniques are still expensive and complex to be used as screening tests [6].

The polymerase chain reaction (PCR) is one of the most sensitive molecular biology techniques as it can detect even minimal quantities of HPV-DNA (as few as one virus genome copy in 100,000 cells) [7].

The PCR technique allows HPV presence to be detected in about 93% of cases of neoplastic lesions of the portio [8].

The cytologic, histologic and molecular biology testing of samples generally leads to an agreement of results in the case of high grade intraepithelial or invasive neoplastic lesions. On the other hand, disagreement has been found in attempts to correlate these methods in the study of low grade lesions [9].

In the present study different instrumental, clinical and laboratory test methods were examined individually and then compared. Lastly, we attempted to verify whether diagnosis could be more accurate using a combination of diagnostic methods.

Materials and Methods

Over a period of one year, 41 patients positive to colposcopic testing with grade 1 abnormal transformation zone undergoing medically-assisted reproduction at the Second Institute of Gynecology and Obstetrics, University of Rome "La Sapienza", were subjected to colposcopic examination. Patients completed a questionnaire about their medical history and risk factors, such as methods of contraception and sexual life style. Ethical approval and informed consent from eligible women were appropriately acquired.

At the beginning of the colposcopic examination oncocytological sampling was performed, as well as exo-endocervical cytological sampling to identify HPV-DNA, mycoplasms, chlamydia trachomatis, HSV 1-2 and adenovirus.

Colposcopy

The colposcopic examination, biological material sampling and the biopsy were performed in all cases by the same physician (A. L.). Initially the presence of gross lesions, vascular details and opacity of epithelium were evaluated. Then, 5% acetic acid was placed over the cervix and vagina and, at both low and high magnification, the entire cervical and vaginal surface was examined, with emphasis on the details of the transformation zone. The investigation was completed using Lugol's solution to identify the extent of the lesion and the grade of the inflammation. Colposcopic patterns were classified according to the 1987 Italian classification [10]. In all cases the colposcopic finding identified was abnormal transformation zone (ANTZ) of grade 1.

All the patients underwent biopsy of the portio under colposcopic guidance.

Processing of clinical specimens

Cervical swabs, taken by Ayre spatula and cytobrush, were seeded in Mycoplasma IST medium (Biomérieux) for *M. hominis* and *U. urealyticum* detection or dissolved in 1 ml of sterile phosphate saline buffer for polymerase chain reaction (PCR) assays. Samples for PCR were centrifuged at 15,000 rpm for 30 min and the pellet, resuspended in 200 µl of PBS, was extracted by the QIAamp Tissue Kit (QIAGEN) according to the manufacturer's instructions. DNA concentration was assessed by optical density determination (260-280 nm) or by electrophoresis on 1% agarose gel with visualisation under U.V. after ethidium bromide staining. Aliquots of the DNA obtained were used in PCR assays for the detection of *C. trachomatis, M. genitalium*, HSV1, HSV2, adenovirus and 45 different HPV genotypes. PCR amplifications were performed on a GeneAmp PCR System 2400 (Perkin-Elmer Cetus, Emeryville, CA). Each DNA sample was first checked for suitability for PCR analysis by amplification of HLA gene sequences using primers GH 26 and GH 27 for HLA Dq Alpha locus (Synthetic Genetics, San Diego, CA). All experiments were performed in parallel with positive and negative controls and under stringent experimental conditions to avoid contamination by "carry over" of DNA.

M. genitalium detection by PCR

The PCR amplification was performed according to Jensen *et al.* [11]. MgPa-1 (5' AGTTGATGAAACCTTAACCCCTTGG-3') and MgPa-3 (5' CCGTTGAGGGGTTTTCCATTTTTGC-3') primers coding for the 140-kDa adhesion protein gene were used to amplify a fragment of 281 bp.

C. trachomatis detection by PCR

The assay was carried out by both a commercial PCR diagnosis kit (by alphagenics) that uses two primers to amplify a segment of 517 base pairs of *C. traschomatis* plasmid DNA and an in-house PCR assay which uses a primer set, plasmid targeted as well, but amplifying a rather small fragment (201 bp). The primers used for generating the 201-bp fragment of the *C. trachomatis* cryptic plasmid were CTP1 (plus strand; 5'-TAG-TAACTGCCACTTCATCA-3') and CTP2 (minus strand; 5'-TTCCCCTTGTAATTCGTTGC-3'). The PCR amplification was performed according to Lan *et al.* [12].

HPV detection by PCR

The use of primers able to promote the amplification of a 526-594 bp fragment spanning the E1 open reading frame (ORF) of genital HPV 6-11-16-18-31 and 33 has been described previously [13]. At the end of the PCR, 10 µl of each amplification product were electrophoresed on 1% agarose gel in parallel with those obtained from cloned HPVs and visualised under U.V. by ethidium bromide staining for detecting HPV-DNA.

Adenovirus detection by PCR

The PCR method according to Allard *et al.* [24] was used for diagnosing adenovirus infections. A specific target DNA sequence (308 bp) was amplifed by PCR using the hexon region primers hexAA1885 (5'-GCCGCAGTGGTCTTACATGCA-CATC-3') and hexAA1913 (5'-CAGCACGCCGCGGATGT-CAAAGT-3').

Cytologic findings have been reported using the Bethesda System while the histologic results have been subdivided into three groups: negative patterns, finding of koilocytosis, condyloma and CIN1.

Cytologic and histologic evaluation were performed by two different operators whose results proved to be in agreement: the data obtained enabled 41 subjects to be taken into consideration as far as the cytologic evaluation was concerned, and 37 for the histologic evaluation.

In order to clarify the role played by each single parameter in the "final" diagnostic definition the correlation among the various morphological and laboratory tests performed was determined.

Results

The presence of infections in the cervicovaginal samples taken was found to be positive for HPV-DNA in 16 cases, for adenovirus in two, and for HSV2 in only one case. Mycoplasmas (*Ureaplasma urealyricum* < 10,000 UCC) were found in 13 patients and chlamydia trachomatis in none (Table 1).

The correlation between the cytologic results and PCR testing for HPV-DNA, as determined in the 41 cases examined, showed agreement in 65.8% of the cases (Table 2). Cytology proved positive for low grade SIL/HPV in 15 cases and for ASCUS in three cases, while it was negative in the remaining 23 cases (Table 1).

Among the discordant positive cytologic results with negative PCR, the cytologic test in seven cases of eight showed up cytonuclear alterations typical of CIN or cytopathic alterations for HPV (Koilocytosis dyskeratosis) and in remaining one sample alterations typical of ASCUS (Table 3).

In the discordant negative cytologic results positive for HPV by PCR, the genotypes identified were always 6 and 11. Furthermore, in 83% of these cases the testing for co-infections was positive (the test for mycoplasmas revealed the presence of *Ureaplasma urealyticum* in 4/6 patients, while only one case was positive for adenovirus) (Table 3).

Table 1. — *Cytology and infections.*

Patient	Cytology	HPV	HSV 1,2	Adenovirus	Mycoplasmas	Chlamydia
A. E.	SIL LG/HPV	–			UU<10,000	
C. P.	SIL LG/HPV	–				
E. A.	SIL LG/HPV	6/11			UU<10,000	
G. E.	SIL LG/HPV	–				
G. P.	SIL LG/HPV	6/11				
O. B.	SIL LG/HPV	–				
R. A.	SIL LG/HPV	6/11/53				
C. A.	SIL LG	16				
M. L.	ASCUS	–				
Me. L.	ASCUS	6/11			UU<10,000	
Or. B.	ASCUS	18		+	UU<10,000	
C. C.	SIL LG/HPV	–				
Ci. C.	SIL LG/HPV	–				
Ga. E.	SIL LG/HPV	6/11			UU<10,000	
R. L.	SIL LG/HPV	6/11/66				
R. A.	SIL LG/HPV	–			UU<10,000	
R. S.	SIL LG/HPV	16				
V. S.	SIL LG/HPV	16				
C. A.	Negative	6/11				
G. S.	Negative	6/11			UU<10,000	
M. S.	Negative	6/11			UU<10,000	
M. C.	Negative	6/11			UU<10,000	
P. E.	Negative	6/11		+	UU<10,000	
S. S.	Negative	6/11				
Ve. S.	Negative	–	HSV 2+			
Ca P.	Negative	–			UU<10,000	
P. C.	Negative				UU<10,000	
O. R.	Negative	–				
M. M.	Negative	–			UU<10,000	
C. S.	Negative	–				
D. C.	Negative	–				
S. C.	Negative	–				
O. M.	Negative	–				
V. A.	Negative	–				
G. V.	Negative	–				
F. T.	Negative	–				
F. S.	Negative	–			–	
F.M.	Negative	–				
Se. S.	Negative	–				
B. T.	Negative	–				
V. C.	Negative	–				

Table 2. — *Correlation between cytologic results and PCR testing for HPV-DNA.*

	PCR Testing for HPV-DNA		
Cytology	Positive	Negative	Total
Positive	10 (*) 4 U.U.+	8 (*) 2 U.U.+	18
Negative	6 (*) 4 U.U.+	17	23
Total	16	25	41

(*) Cases positive for *Ureaplasma Urealyticum*, U.U.<10,000 UCC.

The correlation between histology and PCR for HPV-DNA showed agreement in 51.4% of cases (Table 4). In 14 cases histology proved negative, and positive in 23 cases (for koilocytosis, condyloma or CIN 1). Out the cases proving negative to histologic examination, 35.7% were positive to molecular analysis for HPV: these consisted of bioptic findings described as immature squamous metaplasia tending towards maturation. In all cases virus typing revealed genotypes having a low risk of oncogenic transformation (Table 5).

Table 3. — *Discordant cytologic results and co-infections.*

Cases	Cytology	PCR for HPV-DNA	Co-infections
1	Ascus	Negative	2/8 cases positive for U.U.<10,000 UCC
7	SIL LG/HPV	Negative	
6	Negative	Positive 6/11	4/6 cases positive for U.U.<10,000 UCC 1/6 cases positive for Adenovirus

Table 4. — *Correlation between histology and PCR testing for HPV-DNA.*

	PCR for HPV-DNA		
Histology	Negative	Positive	Total
Negative	9	5	14
Positive	13	10	23
Total	22	15	37

Table 5. — *Histology and infections*

Patient	Cytology	HPV	HSV 1,2	Adenovirus	Mycoplasmas	Chlamydia
A. E.	CIN 1	–			UU<10,000	
C. P.	KOILOCYTOSIS	–				
E. A.	KOILOCYTOSIS	6/11			UU<10,000	
G. E.	KOILOCYTOSIS	–				
G. P.	NEGATIVE	6/11				
O. B.	CIN 1	16				
R. A.	KOILOCYTOSIS	–				
C. A.	NEGATIVE	6/11			UU<10,000	
M. L.	KOILOCYTOSIS	18			UU<10,000	
Me. L.	KOILOCYTOSIS	–				
Or. B.	KOILOCYTOSIS	–				
C. C.	KOILOCYTOSIS	6/11			UU<10,000	
Ci. C.	KOILOCYTOSIS	6/11/66				
Ga. E.	KOILOCYTOSIS	–			UU<10,000	
R. L.	KOILOCYTOSIS	16				
R. A.	CIN 1	16				
R. S.	NEGATIVE	6/11				
V. S.	KOILOCYTOSIS	6/11			UU<10,000	
C. A.	NEGATIVE	6/11			UU<10,000	
G. S.	CONDYLOMA	6/11			UU<10,000	
M. S.	KOILOCYTOSIS	6/11			UU<10,000	
M. C.	NEGATIVE	6/11				
P. E.	KOILOCYTOSIS	–	+			
S. S.	KOILOCYTOSIS	–			UU<10,000	
VE. S.	KOILOCYTOSIS	–	HSV2		UU<10,000	
Ca P.	CONDYLOMA	–				
P. C.	KOILOCYTOSIS	–			UU<10,000	
O. R.	KOILOCYTOSIS	–				
M. M.	NEGATIVE	–				
C. S.	NEGATIVE	–				
D. C.	NEGATIVE	–				
S. C.	NEGATIVE	–				
Q. M.	NEGATIVE	–				
V. A.	NEGATIVE	–			–	
G. V.	NEGATIVE	–				
F. T.	NEGATIVE	–				
F. S.	NEGATIVE	–				

The presence of adenovirus observed in two patients was associated with the simultaneous presence of both HPV-DNA and *Ureaplasma urealyticum*.

Type 2 herpes virus was found in only one sample, in which in any case, none of the other pathogenic agents tested for was detected. The finding of *Ureaplasma urealyticum* (always in low concentrations) was instead more frequent among patients positive for HPV-DNA (Table 5).

In cases in which histologically minimal alterations (koilocytosis) were detected, a high percentage of negativity for HPV was found (11/18), while in cases of low grade lesions the agreement proved to be 66.6% (Table 5).

In the cytohistologic and molecular comparison, the cytologic findings indicated cytopathic effects due to HPV in about 50% of the cases testing positive in the histological examination and negative to molecular analysis for HPV; in the remaining observations, the cytology agreed with the molecular analysis (Table 6).

The correlation between colposcopic examination (abnormal "low grade" transformation zone) and the histologic examination revealed 65% agreement. In the discordant cases of colposcopy testing positive for abnormal transformation zone grade 1 and negative histology, the HPV-DNA test proved positive in 30.7% of the samples, revealing the presence of low risk genotypes.

Global agreement among the four analytical methods used was found in only 20% of the cases, all characterized by the presence of DNA of high oncogenic risk HPV.

Table 6. — *Analysis of discordant cases according to 3 different methods.*

No. of cases	Histology	PCR for HPV-DNA	Cytology
11	Koilocytosis	Negative	5 cases positive for SIL LG 5 negative cases 1 case positive for ASCUS
1	Condyloma	Negative	Negative
1	CIN1+HPV	Negative	SILLG
5	Negative	Positive 6/11	4 negative cases 1 case positive for ASCUS

Conclusion

Analysis of our results relative to the "low grade" colposcopic lesions revealed good agreement between the cytologic examination and HPV testing by PCR, comparable with the literature data [17]. The cases with negative cytologic examination, but testing positive to PCR, may be accounted for by latent HPV infection or the presence of a limited number of infected cells containing only a few virus genomes that are insufficient to cause any significant cell modifications.

Conversely, cases testing positive to cytologic examination, but negative for HPV, may be the result of different sample procedures used in the two tests or of the presence of co-infections due to other pathogenic agents (mycoplasmas, adenovirus).

As far as samples testing negative to PCR for HPV and positive to the histological examination for the presence of koilocytes are concerned, it must be borne in mind that the term "koilocytosis" should be reserved for squamous epithelia with nuclear enlargement associated with vacuolization.

Perinuclear halos in the absence of significant nuclear enlargements are frequent in the metaplastic epithelium of the cervix and should not be considered as "suggesting" a diagnosis of HPV infection. Lastly, the finding of HPV positivity through PCR testing in histologically negative samples might indicate those cases of squamous metaplasia with initial low risk HPV infection in which only a particularly sensitive method can successfully detect the presence of virus.

Evaluation of colposcopic-histologic correlation confirms the known low specificity of the colposcopic examination even though it remains a particularly sensitive and valid method for the early diagnosis of low grade cervical lesions.

Overall agreement among the diagnostic methods used in clinical practice is generally found in cases of low grade cervical lesions accompanied by the presence of high oncogenic HPV-DNA.

In conclusion, even though diagnostic methods used separately are not always able to provide complete diagnosis and biological characterization of a low grade cervical lesion, their association may be considered as the current diagnostic "gold standard" in which they are pieced together into a jigsaw puzzle in which their true nature is expressed, especially as far as detecting low grade cervical lesions with potential risk of progression is concerned.

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