Role of the association of high-risk HPV identified by real-time PCR in cervical preneoplastic lesions

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Summary

Objective: To evaluate the effects of infection in multiple types of high-risk human papilloma virus (HPV) in cervical preneoplastic lesions in patients undergoing colposcopy following a diagnosis of atypical squamous cells of unknown significance (ASCUS) and low-grade squamous intraepithelial (LSIL) cytology. *Materials and Methods:* Between 2009 and 2010, 2,500 patients were recruited with a mean age of 35 ± 5 years. Screening for cervical cancer was performed and in case of ASCUS and LSIL the patients underwent colposcopy. The tests for the detection and typing of viral DNA (HPV - DNA test) were performed on cervical swab with real-time PCR amplification. *Results:* The prevalence of infection was 70% (1579/2256) in the patients recruited. In relation to the degree of preneoplastic lesions some high-risk HPV viral genotypes were identified: HPV 16 (319/1466), HPV 18 (164/1466), HPV 45 (76/1466), HPV 31 (215/1466), HPV 52 (145/1466), HPV 58 (55/1466) HPV 56 (79/1466), HPV 51 (110/1466), HPV 6 (138/1466), HPV 11 (88/1466), HPV 42 (34/1466), HPV 53 (43/1466). In case of high-grade lesions of CIN (CIN2 and CIN3) a greater HPV co-infection was detected and in particular the association from 16 to 18 (70%), 16-33 (18%) and 16 to 52 (12%). *Conclusions:* Infection caused by the simultaneous presence of multiple HPV genotypes appears to be associated with a significantly increased risk of high-grade lesions of CIN or invasive cancer than the presence of single viral infections. The infection with multiple HPV types is a significant risk factor for high-grade lesions of CIN in women undergoing colposcopy for ASCUS cytology / LSIL. The use of real-time PCR has shown the ability not only to identify the different types of HPV, but also to monitor quantitatively the same over time, and during the study phase, to evaluate the sensitivity and specificity of the method in comparison with other techniques.

Key words: Squamous cell carcinoma; Endometrial carcinoma; Ichthyosis uteri.

Introduction

Human Papilloma Virus or HPV is a common sexually transmitted infection. About 75% of sexually active women become infected during their lifetime with an HPV of any type, more than 50% infected with a high-oncogenic risk type [1-4]. Although infection may occur without symptoms (latent or subclinical) and can be resolved (immunity cell-mediated), about 40% of cases are associated with high-grade squamous intraepithelial lesion (HSIL). Across all five continents, HPV has been reported to be the most common genotype in high-grade cervical intraepithelial neoplasia (CIN2+) with an incidence rate ranging from 33.3% in Oceania to 51.8% in Europe [5, 6]. The guidelines provide, therefore, that in case of abnormal diagnostic cytology (ASCUS and LSIL), found on screening, make a colposcopy, and/or as an alternative strategy is recommended HPV molecular biology research, while others, advising molecular research as co-colposcopic examination tests [3, 7]. This research allowed the authors to detect the presence or absence of HPV in low- or high-risk virus (16,18,31,33,35, 39,45,51,52,56,58,59,68,73,82). There are several methods of molecular biology and whichever is used, only the presence/absence of the viral genome in cells and tissues, viral genotype, or quantification of the viral load can be identified. The various molecular methodologies are officially recognized, although hibryd capture II (HCII) and polymerase chain reaction (PCR) are most often used [8-12]. The infection caused by the simultaneous presence of multiple HPV genotypes in patients with ASCUS and LSIL cytology is an important risk factor for the emergence of high-grade CIN and squamous cell cervical cancer in women undergoing colposcopy. The purpose of this observational epidemiological study was to determine, through real-time PCR, the simultaneous presence of several high-risk HPV types and their incidence in the presence of high-grade CIN in patients undergoing colposcopy after US cytology ASC and LSIL [13, 14].

Materials and Methods

The study was conducted at the "San Sebastiano and Sant'Anna" Hospital of Caserta and the San Carlo testing center of Caserta. The recruitment of patients occurred prospectively and spontaneously at the Colposcopy Clinic of the Second University of Naples based in the Caserta Hospital. Subjects of the study included 2,500 patients, recruited from a strong response to the authors observation, between the years 2009-2010, with the following inclusion criteria: aged between 18 and 70 years, under screening investigation that resulted positive for ASC-US or LSIL. Exclusion criteria included: confirmed malignant disease, HIV-seropositive patients, chemotherapy or radiation therapy for pelvic diseases even before the recruitment or at any other time during the study period, hysterectomy, inadequate research and HPV cytological reading, and refusal of informed consent. All patients were subjected to: anamnesis, gynecological visit, colposcopy, and biopsy; HPV typing through real-time PCR technique.

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Patients enrolled in the study were 35 ± 5 years old. The Pap test was classified by a pathologist according to the Bethesda System 2001. The colposcopic examination was carried out by two operators according to the criteria of the International Classification of Barcelona 2002 criteria and biopsy was performed in suspicious areas. The tests for the detection and typing of viral DNA (HPV-DNA testing) was performed according to a cervical swab real-time PCR method with amplification, at the San Carlo testing center in Caserta. The real-time PCR allowedfor a qualitative test, a quantitative assessment of genotyping, and viral load, and is a DNA amplification technique based on continuous monitoring of the amplification products over time. This method has high sensitivity and playback speed. Measuring amplification in real-time during the exponential phase of PCR when the efficiency of amplification is minimally affected by the variables of reaction, allows for more accurate results than conventional PCR. With real-time PCR, therefore, the authors were able to monitor progress of the ongoing reaction and the data obtained at the end of the cycle was used to make a relative quantification of the amplified fragment. This is made possible through the use of fluorescent markers which follow the same kinetic accumulation of the PCR reaction. The fluorescence, emitted as a result of a specific radiation from the light source of the thermal cycler is measured in real-time by a charge coupled device (CCD) camera, and is directly proportional to the amount of amplified product. All operations related to the measurement were carried out under the control of a program run by a personal computer. Data processing was performed using SPSS software for Windows.

Results

During the study, 2,500 patients diagnosed with ASCUS/LSIL were recruited; 96% of these (2,400) were submitted to colposcopy, the remaining 4% (100) did not accept continuation in the study. Of the patients undergoing colposcopy, a group of 144 (6%) patients were excluded because they did not fit the inclusion criteria. Of the remaining 2,256 patients, 26% (587) presented with ASCUS cytology and 74% (1,669) LSIL. The pathological diagnosis of lesions of high-level and low-grade CIN was made by biopsy, performed during the course of colposcopy only in 65% (1466) of patients. The 790 samples were not biopsied during colposcopy because, despite having cytologic results with ASC-US and LSIL, colposcopic pictures did not suggest biopsy and histological investigation. The prevalence of histological diagnosis was negative in 34% (498) patients, positive in 43% (630) patients for CIN1 lesions (253 and 377 patients with ASCUS and LSIL, respectively), the percentage of CIN2 was 22.4% (328) of patients recruited (132 and 196 patients with ASCUS and LSIL, respectively), and finally the prevalence of CIN3 or cervical cancer was 0.6% (8) (3 and 5 patients in case of ASCUS cytology and LSIL, respectively) (Figures 1-2). The prevalence of HPV was 70% (1579/2256); 65% (1,466) of patients undergoing biopsy, depending on the degree of pre-neoplastic lesions were identified as high-risk HPV: HPV 16 (319/1466), HPV 18 (164/1466), HPV 45 (76/1466), HPV 31 (215/1466), HPV 52 (145/1466), HPV 58 (55/1466), HPV 56 (79/1466), HPV 51 (110/1466), HPV 6 (138/1466), HPV 11 (88/1466), HPV 42 (34/1466), (Figure 3) HPV 53 (43/1466). The remaining 35% of patients did not have a biopsy because they did not show suspicious lesions during colposcopy examination. Research of the simultaneous presence of multiple viral types demonstrated the role played by the association of high-risk oncogenic HPV (16, 18, 31, 33, 52 and 58) in CIN2 and CIN3. In fact, in the latter (30% of cases), more HPV co-infection was detected, which was expressed mainly with associations 16-18 (70%), 16-33 (18%) and 16-52 (12%). HPV 45 merits different attention, as is clear from recent literature, even if CIN3 are present in a low percentage of high-grade lesions, while more viral cervical adenocarcinoma are present (Figure 4).

Discussion

The results of this study suggest that the infection caused by the simultaneous presence of multiple HPV genotypes in patients undergoing colposcopic examination, as a result of ASCUS or LSIL diagnosis, seems to be associated with a significantly increased risk of high-grade CIN or invasive cancer compared to cases where there is the presence of single viral infections. Since the presence of viral DNA was detected in all cervical cancers, it is clear that the finding of papillomavirus infection, at an early stage, may have a prognostic and predictive value [7]. In fact, this event may result in cellular DNA mutations that cause the appearance of abnormal clones in cervical cells until carcinoma. These biological findings allowed the authors to understand how a high-risk HPV infection contracted at a young age may have a prognostic significance when it appears worse in older age, in the presence of persons with insufficient immunological response, and at greater risk of exposure to sexually transmitted oncogenic cofactors [15]. From this rationale, is important to seek the presence of viral DNA in the lower female genital tract. Conventional cervical screening has greatly reduced the mortality of cervical cancer, but this method is inadequate and is responsible for a significant percentage of false negatives that can not be eliminated and can reach 10%. Furthermore, cytologic shortcomings may generate higher false negatives in pre-invasive forms, where the preventive role is very important compared to the invasive form. These deficiencies are due to the fact that the Pap test identifies abnormal cellular activity produced by the virus or histological pathologies that absolutely need to be confirmed by histological diagnosis. For viral infection diagnosis, HPV testing result is instead much more sensitive. In addition, the test can be used to highlight the simultaneous presence of different viral types, as most recent literature shows [16], which are correlated with high-risk injury. In fact, women with persistent HPV infection or co-infection of high-risk types have more than 300 times higher risk of developing CIN compared to women who test negative. From these observations comes the proposal to integrate HPV testing with cervical screening, as this combined testing would significantly reduce the mor-

Fig. 1

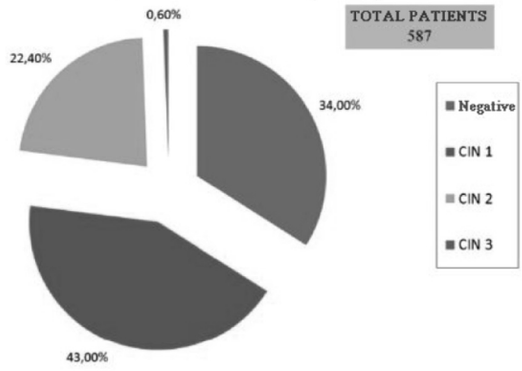

Figure 1. — Percentage of case studies (ASCUS).

Fig. 2

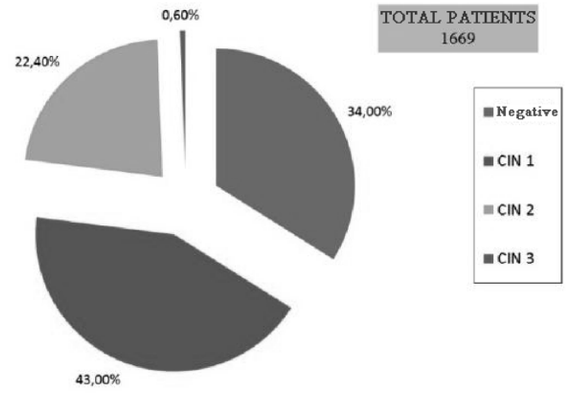

Figure 2. — Percentage of case studies (LSIL).

Fig. 3

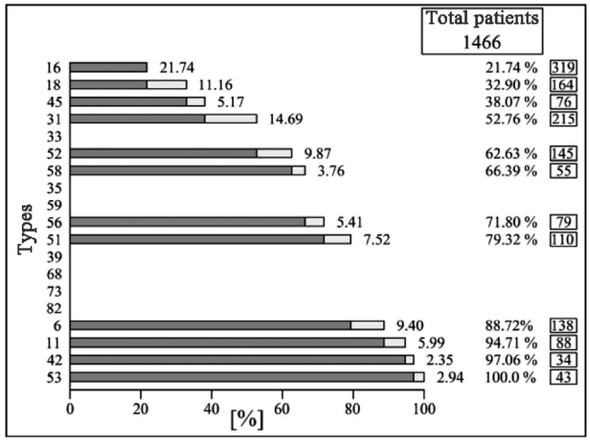

Figure 3. — HPV types in cervical cancer.

Fig. 4

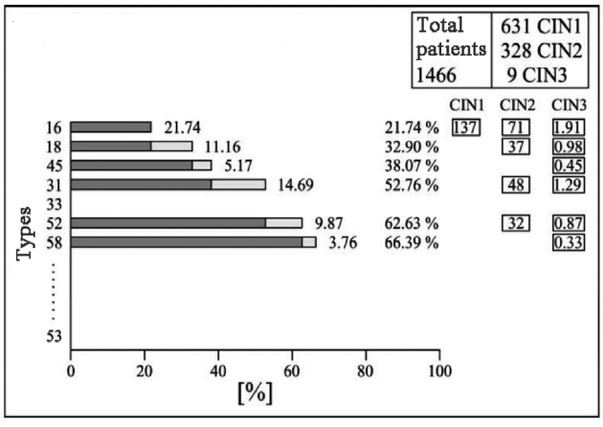

Figure 4. — HPV types in cervical cancer

tality of cervical cancer [7]. In addition, HPV-DNA testing could have a rational in clearing doubts in cases of low-grade cytology or histology in order to establish an appropriate treatment program or follow-up. The test is also used to determine the persistence of infection after treatment for CIN (conization or LEEP) or in cases where the colposcopic lesion shows a normal Pap test. However, there still remains the difficulty of choosing which method is better in terms of sensitivity and specificity for the detection and typing of HPV. There are various molecular methods officially recognized, although those most often used are HCII and PCR [8, 10, 17, 18]. The real-time PCR used measures the amplification in real time during the exponential phase of the development of the method, when the amplification efficiency is affected minimally by the variables of reaction, allowing for more accurate results compared to traditional PCR. Then the progress of the reaction can be monitored while it is still in progress, and the data obtained at the end of the cycle can be used to make a relative quantification of the amplified fragment. This is possible through the use of fluorescent markers which follows the same kinetic accumulation of the PCR reaction. The fluorescence, emitted as a result of a specific radiation from the light source of the thermal cycler, is measured in real-time by a CCD camera and is directly proportional to the amount of amplified product. This also allows the degree of viral replication to be obtained by calculating the logarithm of the concentration of HPV in 100,000 human cells, with the following clinical interpretation: < 3 clinically insignificant or little meaning; evaluated clinically by 3-5, the possible risk of dysplasia; > 5 clinically important, high- risk for dysplasia.

The above differs substantially from other methods currently used, in fact: 1) The HCII is a qualitative test and semi-quantitative test, which uses the reaction between HPV DNA, if present, and specific RNA probes, with the formation of hybrid DNA / RNA in the liquid phase, which are captured on solid phase by an Ab universal microplate and finally detected by a chemiluminescent substrate. This test can identify low-risk genotypes 5 (6,11,42,43) and 13 high-risk (16,18,31,33,35,39, 45,51,52,56,58,59,68) [17, 18]. The multiplex PCR agarose gel analysis allows more targets simultaneously by using mixtures of primers or degenerate primers. It

provides for the identification of 12 high-risk genotypes (16,18,31,33,35,39,45,52,56,58,59,66) and two low-risk, it is theoretically possible highlight all genotypes [17, 19]. 3) PCR + Reverse Dot Blot refers to the technique by which they are transferred to solid matrix (nitrocellulose and/or nylon) small molecules of DNA, the order of a few hundreds of bases, the test has a sensitivity of 50/100 genomes, and allows a comparative analysis of immediate results [17, 19]. The use of nucleic acid amplification techniques using real-time PCR, a method used in this study because it was considered most suitable, advantageously allowed to identify with high sensitivity and specificity of the viral sequences present in very small amounts of a biological sample and quickly results in a high number of copies of a specific DNA fragment. This method saves a lot of time, reducing the time required for the report (turn around time), also being a closed system it decreases the chances of contamination, thus obtaining an accurate and sensitive diagnosis compared to traditional methods of PCR, also allowing the identification of resistant mutations. However, among the disadvantages are high costs for necessary equipment and high technical knowledge [17, 19]. The authors can state that the biological testing of molecules, used for viral typing is of fundamental importance in the diagnosis of infection and in screening for cervical cancer, because this method is much more effective compared to others as it allows to evaluate not only the presence/absence of HPV, but also the identification of the genotype and the degree of replication-values that are also useful in follow-up colposcopy. The current was also an observational study of the method, that aimed to evaluate the cytological context, the presence of colposcopic lesions, and viral infections by one or more types of HPV and histological lesions related to them at time zero with real-time PCR. This study has a limit as it would have been appropriate to include patients in a program of close follow-up to verify and confirm, in the presence of multiple HPV infection, the possible evolution in high-grade lesions, by monitoring the quantitative value of the method to identify whether it can play a role for the observation of the virus permanently and/or its disappearance. Another limit of this method was that it was still possible to perform a comparative study with other methods of molecular biology for the detection of HPV, as already reported in the literature. In this regard, another study at the same laboratory and on the same population will include a comparative examination of real-time PCR and agarose gel electrophoresis; the investigation is still ongoing. The association between multiple HPV infection and an increased risk of cervical precancer lesions has already been reported in a prevalence of studies [20, 21]. On the contrary, the effect of multiple infections on the incidence of high-grade CIN seems more controversial.

Wheeler *et al*. [22] analyzing data from the ALTS trial, failed to show a consistent interaction between multiple infection and incident high-grade CIN. One possible explanation for these conflicting results could be the different characteristics of prevalent and incident HPV infection. Multiple HPV infection is associated with a noticeably increased infection duration which, in turn, could increase the risk of CIN [23]. As the duration of infection increases, its chance of detection as a prevalent case increases, causing an over-representation of severe infection in prevalent studies. Additional longitudinal investigations in larger populations will be necessary to clarify this issue.

Although the ratio is much lower for ASC-US/LSIL as reported in the literature, in the authors' laboratory, the relationship seems to be reversed, as also reported by Spinillo *et al*. [24].

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