"Low-grade positivity" of HPV viral load after atypical squamous cells of undetermined significance (ASC-US) cytology identifies women at low-risk for cervical intraepithelial neoplasia grade 2 and 3

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Summary

The correlation between high-risk HPV-DNA viral load, expressed as relative light units (RLU) values obtained from the Hybrid Capture 2 (HC2) test, and the prevalence of CIN2/CIN3 was investigated and statistically analyzed in 614 ASC-US consecutive cases. Cases were categorized into three groups according to RLU values: "low-grade positivity", "intermediate positivity" and "high-grade positivity", and the prevalence of CIN2/CIN3 was evaluated in the single groups and compared among them. CIN/CIN3 rates demonstrated a significant ($p < 0.001$) increase with a direct correlation with increasing RLU values: 4.6% (RLU from 1.0 to 10.0), 9.1% (RLU from 11.0 to 100.0) and 32.2% (RLU > 100.0) respectively. The prevalence of CIN2/CIN3 between the group with RLU < 10.0 (4.6%) and the group with RLU > 10 (24.2%) showed statistical significance ($p = 0.0002$). Increasing hrHPV viral load significantly correlates with increasing prevalence of CIN2/CIN3 in ASC-US cases.

Key words: ASC-US; HPV; HPV-DNA; viral load; HC2; Cervical Intraepithelial Neoplasia; CIN.

Introduction

Uterine cervical cancer is the second most common malignancy among women worldwide [1], and its incidence has demonstrated a dramatic decrease in response to widespread cytology-based (Pap test) screening programs [2]. Since the introduction of the 2001 Bethesda system (TBS) for cervical cytology, the category of atypical squamous cells of undetermined significance (ASC-US) represents the commonest abnormal cytological result in many countries [3-5], accounting for almost two million cases per year in the United States. According to the well documented cause-effect role of high-risk human papillomaviruses (hrHPV) in cervical oncogenesis [6], hrHPV-DNA detection has been demonstrated to significantly improve the low sensitivity of conventional cytology and has been clearly identified as the highest performance option for the triage of ASC-US cases [7]. In this setting, the sensitivity of hrHPV-DNA testing for the detection of high-grade cervical intraepithelial neoplasia (CIN2/CIN3) is reported as 83%-100%; this high sensitivity is however often correlated with low specificity (63%) and low positive predictive value (PPV) [8], determining too high referral rates to second-level colposcopy and biopsy compared to the low prevalence of true lesions. In fact, the rate of CIN2/CIN3 in ASC-US cases is reported as high as 15-17% [9]. For this reason, many attempts have been made to improve the tests specificity and optimize the performance of the ASC-US triage with the viral tests, the goal being the identification of the subgroup of patients with an underlying histology-proven CIN2/CIN3 lesion; in this field, hrHPV viral load quantification represents an interesting issue and has been diffusely investigated in different settings [10-12], the consistent results of these studies being the significant direct correlation between increasing hrHPV viral load and increasing risk of worsening cervical lesions. The detection of the viral load in biological samples is achievable by the use of different biomolecular laboratory techniques (real-time PCR, bDNA, NASBA) with most of them being particularly sophisticated, expensive, and high-expertise correlated; due to this, these approaches have very little or no use in clinical practice. Hybrid Capture 2 (HC2) is one of the most widely adopted hrHPV-DNA tests worldwide. Together with the positivity/negativity result in a qualitative fashion, the test also allows an indirect quantification of the hrHPV-DNA copies detected in the collected cervical sample. Assuming that qualitative hrHPV-DNA testing is validated as the most effective triage option of ASC-US cytology, in this study, we tested the hypothesis that different levels of hrHPV-DNA viral load in these cases might correlate with different risks of an underlying CIN2/CIN3. In particular we investigated if low levels of hrHPV-DNA viral load might reduce the risk of high-grade CIN.

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Materials and Methods

The study population is represented by 614 consecutive and unselected patients from the greater Milan area, screened at San Raffaele Scientific Institute and European Institute of Oncology (IEO) in a 2-year period, who were cytologically diagnosed with ASC-US. All cases underwent high-risk HPV-DNA (hrHPV) testing using the commercially available HC2 assay (Qiagen Inc. Corporation, Germany) following the manufacturer's instructions for sample collection and result interpretation.

Testing for the DNA of oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 was performed on 4 ml aliquots of the PreservCyt samples. The test involves denaturalization, hybridization of HPV target DNA with a cocktail of full-length HPV type specific RNAs followed by capture of DNA/RNA hybrids on a solid phase and amplification of the signal by the binding of hybrids to multiple conjugated antibodies that specifically recognize DNA/RNA hybrids. The reaction is chemoluminiscent and is detected by a luminometer, which provides relative quantification of each individual sample compared with the mean of a series of positive controls containing 1.0 pg/ml HPV DNA; the latter is identified as the cut-off of positivity (CO). Results are quantified as the ratio between relative light units (RLU) of the tested sample and the positivity cut-off. (RLU/CO). Accordingly, a RLU/CO value equal or greater than 1.0, corresponding to 5000 or more HPV-DNA copies per test is considered positive.

RLU/CO values from each sample were collected and recorded and, for the purpose of the study, categorized in three groups of increasing values: samples with ratios ranging from 1.0 to 10.0 were defined as "low-grade positivity", cases ranging from 11.0 to 100.0 RLU/CO were defined "intermediate positivity", and cases with RLU/CO > 100.0 as "high-grade positivity".

According to guidelines for the triage of ASC-US cytology, hrHPV-DNA positive cases (RLU/CO > 1.0) were referred to second-level colposcopy and, if indicated by aceto-whitening areas or unsatisfactory colposcopy, a biopsy/endocervical curettage was performed and histologically examined. In the event of negative and satisfactory colposcopy, a repeat Pap test was immediately obtained and considered diagnostic.

According to pathological diagnosis, cases were classified as negative, low-grade squamous intraepithelial lesions (HPV/CIN 1) or high-grade squamous intraepithelial lesions (CIN2/CIN3).

Correlation between final diagnosis and RLU/CO groups was studied and, in particular, the prevalence of CIN2/CIN3 lesions among the three categories of HC2 load positivity was compared.

Results were statistically interpreted with descriptive analysis, Fisher's exact and chi-square test with a 95% confidence Interval (CI) (software: IBM SPSS Advantage for Microsoft Excel); an alpha value < 0.05 was regarded as significant.

Being a retrospective study no power calculations were priorily performed as exploratory statistical analysis was the goal. As no other tests were being performed than routine and validated protocols for ASC-US cytological diagnosis, the Institutional Review Board gave exemption to ethical approval, and informed consent was obtained only for data collection and analysis.

Results

Out of the 614 ASC-US cases that underwent HPV-DNA testing, 338 (55%) had a negative result (hr-RLU/CO < 1). The remaining 276 (45%) patients with a positive HC2 test were colposcopically triaged and represent the study group.

Mean and median age of the studied cases were 38.3 and 37.6 years, respectively. Time from HPV-DNA testing to outcome final diagnosis ranged from 0 to 12 months, with a mean of 3.4 months.

Among the 276 cases with a clinical follow-up, 92 (33.3%) had a negative final diagnosis, 134 (48.5%) were classified as low-grade lesions (HPV/CIN1) and 50 (18.1%) as high-grade lesions (CIN2/CIN3).

According to RLU/CO ratio results of HC2 and to the study purpose, 86 (31.1%) patients were classified as "low-grade positivity" (> 1.0 ≤ 10.0), 66 (23.9%) as "intermediate positivity" (≥ 11.0 ≤ 100.0) and 124 (44.9%) as "high-grade positivity" (> 100.0) of RLU/CO ratios.

Out of the 86 "low-grade positivity" cases, 38 (44.2%) were negative, 44 (51.2%) had CIN1 and four (4.6%) had CIN2/CIN3; in detail, these four cases showed an RLU ratio ranging from 4.9 to 6.9. The corresponding prevalence of CIN2/3 in the other two groups of RLU ratios was 9.1% and 32.2% for "intermediate" and "high-grade positivity", respectively (chi-square: $p < 0.001$ – CI 95%); combining these latter two groups (RLU/CO from 11.0 to 100.0 and over 101.0), the cumulative risk of CIN2/3 was 24.2% (46/190), which was a significantly greater risk than that of women with 1.0 to 10.0 RLU/CO ratios (4.6% - 4/86) (chi-square and Fisher exact test: $p = 0.0002$ – CI 95%).

For patients with a negative follow-up outcome (92 cases) or affected by CIN1 (134 cases), no statistically significant differences were observed among the three groups of RLU/CO (chi square: $p = 0.29$ - CI 95%) and between women with "low-grade positivity" (RLU/CO from 1.0 to 10.0) and women with > 10.0 RLU values (chi square: p = 0.73 - CI 95%). Results are graphically summarized in Table 1.

Table 1. — *RLU/CO groups and clinical outcome*.

Outcome	No. of cases	RLU/CO groups 1-10	11-100	> 101
Negative	92	38 (44.2%)	22 (33.3%)	32 (25.8%)
CIN1	134	44 (51.2%)	38 (57.5%)	52 (41.8%)
CIN2/CIN3	50	4 (4.6%)*	6 (9.1%)	40 (32.2%)
Total	276	86	66	124

RLU/CO = relative light unit/cut-off; CIN = cervical intraepithelial neoplasia.
*$p < 0.001$.

Discussion

The overall prevalence of histologically confirmed CIN2/CIN3 lesions in our study group of hrHPV-DNA positive ASC-US women (50 cases - 18.1%) is only apparently beyond the expected rates of a screening setting that, accordingly with reported data from large series, should range from 10-17% [3, 9]. In our opinion this can be explained by the age characteristics of our study population; in fact, mean and median age were 38.3 and 37.6 years, respectively. This age group can be easily recognized as a high-risk group for viral persistence

and/or precancerous progression [13], with a significant increase of CIN2/CIN3 detection rate compared to younger age groups [10, 13]. Moreover, in young patients a significantly higher percentage of spontaneous viral clearance and intraepithelial neoplasia (CIN) regression to normality is well documented [14]. Accordingly, our study group appears to be particularly suitable to be investigated from the virological side.

HPV viral load detected by the use of HC2 has already been reported by several studies as directly correlated with CIN2/CIN3 or cervical cancer frequency [10, 12, 15, 16]. However, the ASC-US cytological category has been poorly investigated since the validation of hrHPV-DNA testing is the optimal triage option in these cases compared to repeat cytology and immediate colposcopy [7]. In fact, despite the high sensitivity of the viral tests in identifying the real negative cases, the specificity remains poorly satisfactory and leads to high referral rates to second-level colposcopy and histology [3, 9, 17]. The first noteworthy result of our study is consistent with our tested hypothesis: increasing HPV viral load expressed as RLU/CO ratios in ASC-US cases is significantly correlated with increased prevalence of CIN/CIN3 lesions ($p < 0.001$). In our opinion, this is particularly relevant because it can be seen both in terms of stratifying the risk of CIN2/CIN3 and as a viable option to improve hrHPV-DNA testing specificity. The issue of improving the test specificity has already been considered by previous papers, suggesting and testing higher RLU/CO cut-off points of positivity: the majority of these experiences concluded that raising the cut-off of positivity (e.g., to 2.0 RLU/CO) determined a loss of sensitivity [18, 19]. One of the major positive characteristic of HC2 is reproducibility, also for low levels of positivity. However, several studies suggested that RLU/CO levels around the cut-off of positivity may be considered false-positives due to cross-contamination of samples or chemiluminescent signals in adjacent wells [20, 21]. For this reason the manufacturer's recommendations include retesting samples with borderline results (RLU/CO between 1.0 and 2.5) and reporting as definitely positive a sample with a retest of 1.0 or above. In case of a retest with less than 1.0 RLU/CO a second retest should be performed, with the results of the third test being the final result.

In the current study we demonstrated that ASC-US cases with RLU/CO ratios below 10.0 are associated with a significantly lower rate of histologically proven CIN2/CIN3 compared to RLU/CO ratios > 10.0 (4.6% vs 24.2% - $p = 0.0002$); we did not demonstrate any difference in CIN1 prevalence according to RLU/CO ratios. Our results are consistent with those of Jarboe *et al*. [22], who recently reported 3.2% and 17.3% ($p = 0.047$) in the same groups respectively, and no difference in CIN1 prevalence; this is, to our knowledge, the one and only paper that approached ASC-US cases in this fashion. The authors concluded that in weakly hrHPV-DNA positive ASC-US cases a modification of the management algorithm may be justified. In particular, referral to colposcopy could be hypothesized for cases with RLU/CO above 11.0. This appears to be a very interesting proposal, and our results reinforce the validity of this option. Moreover, differently from the cited paper in which no information about patient age was available, our results came from a high-risk subgroup of patients from the age-related standpoint (mean age 38.3 yrs), and thus seem to be bias-free. We do agree that a modification of the standard algorithm for the triage of ASC-US [7] based on quantitative results from the HC2 assay would need to be carefully considered [22], and that other options such as HPV genotyping [23] or the use of new biomarkers ($p16^{ink4}$/Ki-67) [24] deserve further investigation in the same field of application. Nonetheless we feel that the biomolecular viral aspects of cervical cancer precursors in terms of early detection are particularly noteworthy and promising.

Conclusion

The detection of high-risk human papillomavirus viral load expressed as RLU results of the HC2 test correlates with the prevalence of histologically confirmed CIN2/CIN3 lesions after ASC-US cytology. These results may allow modifications of the triage algorithm for these cases.

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