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Immediate risk of HSIL presence in women who have both ASC-US cytology and negative high-risk HPV test

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Summary

Purpose: The authors aimed to detect immediate risk of having high grade squamous lesions (HSIL) in atypical squamous cells of uncertain significance(AS-CUS) and concomitant high-risk human papillomavirus (HrHPV) testing as negative [HrHPV(negative)AS-CUS]. *Materials and Methods*: The authors performed immediate/baseline colposcopy on concomitant HrHPV (negative)AS-CUS cases. Pap tests were evaluated with liquid-based cytology (LBC) and HrHPV detection was performed in LBC material with PCR. Colposcopic diagnoses and biopsy results were compared with Pap test and HrHPV test results. *Results*: There were 104 patients over a one-year period. In all, 84 cases were included. Colposcopic biopsies revealed low grade squamous intraepithelial lesion (LSIL) in 19 cases (23%) and HSIL in three cases (4%). Intrauterine device use and smoking were significantly correlated with presence of HSIL ($p = 0.005$ and $p = 0.007$ respectively). *Conclusion:* Similar data in literature, 4% of ASC-US-HrHPV (negative) cases are expected to have HSIL in follow-up periods less than six months. The present authors believe clinicians should be more open with their patients about limitations of Pap-HPV testing.

Key words: ASC-US; Cervical cancer screening; Human papillomavirus; Human papillomavirus DNA tests.

Introduction

After its introduction in 1990's, the term atypical squamous cells of uncertain significance (ASC-US) has been accepted worldwide and today, atypical squamous cells (ASC) is one of the most popular diagnoses in cervicovaginal smear (CVS) assessment. In the USA, more than two million cases of ASC per year are diagnosed in CVS's [1]. The frequency of ASC, although varies among laboratories, and is between 0.8% to 1.4% in the present authors' country which is Turkey [2]. Although majority of patients with ASC diagnosis are turned to have a benign reactive process, a considerable subset of women harbor squamous intraepithelial lesion that may progress to invasive cervical carcinoma [3].

Current guidelines recommend testing high-risk human papillomavirus (HrHPV) in triage and management of ASC cases [4]. In these recommendations, whereas HrHPV(+)/ASC cases usually is being to advised to have colposcopic examination, HrHPV(-) ASC-US cases are advised to follow up in specific periods of time. This duration is varied among different guidelines. The current study aims to investigate baseline real frequency of HSIL (cervical intraepithelial neoplasia, CIN2-3) in HrHPV(-) ASC cases.

Materials and Methods

Study design and patient selection

Approval was granted for the present study from the Local Ethics Committee (07.06.2012). All women who accepted to take part gave written informed consent before enrollment to the study. Current evaluation focus on data from women who underwent immediate colposcopy in three months after their ASC diagnosis in CVS and negative HrHPV test in same liquid based cytology material.

Study was carried over a one-year period. Patients who applied for annual Pap smear screening were included. Cervical cytological screening was made with liquid-based cytological method (ThinPrep Pap test). Cytological screening results were classified according to The Bethesda System 2001 (TBS 2001). Cases whose smear result were ASC-US were subjected to HrHPV detection and genotyping test. ASC-H (ASC-US ruled out HSIL) cases were not included in current research. In this particular assessment, all women with ASC diagnosis were scheduled for colposcopy unless an contraindication was present in considerable time (usually in three months) regardless of their HrHPV test.

HrHPV DNA test

As a routine application, the present authors utilized a qualitative multiplex assay that provides specific genotyping information for HPV types 16 and 18, while concurrently detecting the other 12 HrHPV types in a pooled result. β-globin from cellular input was used as an internal control to assess specimen quality and identify specimens containing factors that inhibit the amplification process. This test is a qualitative in vitro test for the detection of HPV in patient specimens. The test utilizes amplification of target deoxyribonucleic acid (DNA) via polymerase chain reaction (PCR) and

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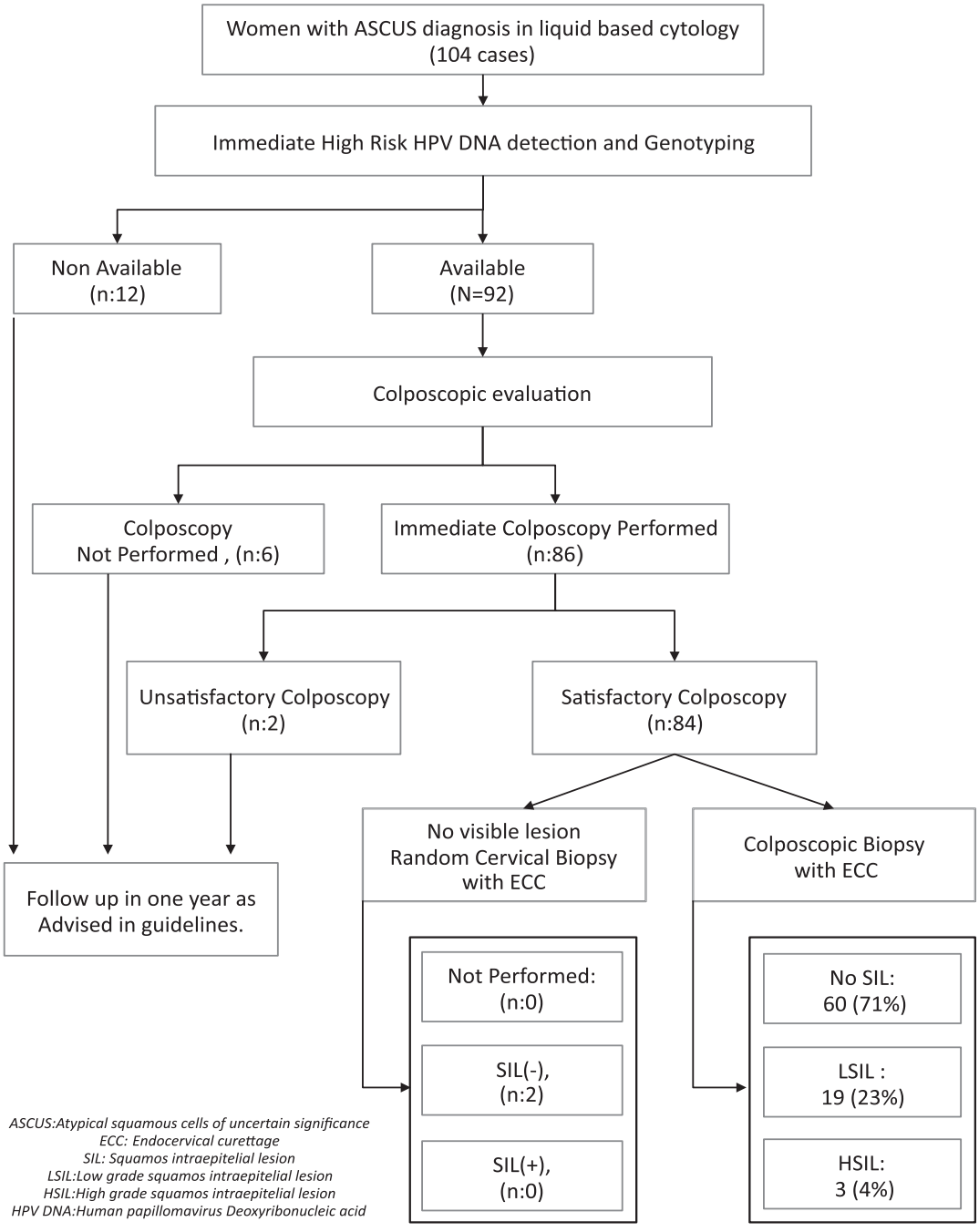

Figure 1. — Flow chart of HrHPV negative ASC-US cases.

nucleic acid hybridization for the detection of 14 HrHPV types in a single analysis. The test specifically identifies (types) HPV 16 and HPV 18 while concurrently detecting the rest of the high-risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) at clinically relevant infection levels.

Colposcopic evaluation

Data from those who were, pregnant, postmenopausal, previously being followed with a diagnosis of abnormal cervical smears, had vaginal bleeding during the procedure, had used vaginal creams within the last month, had sexual intercourse within the last two days prior to the colposcopy examination, have been excluded from the study. Leisegang Berlin West Type Fbw Germany) was used for colposcopic evaluation. After inserting the speculum, cervix was flushed with saline and then all the squamocolumnar junction and transformation zone were observed using a x40 magnification. Afterwards, 3% acetic acid solution was applied to cervix. Colposcopic biopsy was performed when necessary. Random biopsy and endocervical sampling were performed when no lesion was observed during colposcopy.

Normal colposcopic findings were considered as original squamous epithelium, columnar epithelium, and normal transformation zone. Acetone white spaces, punctuation, mosaicism, iodine negative epithelial tissue, leukoplakia, and atypical vascularia-

Table 1. — *The demographic characteristics of the participants.*

	Number	Percentage (%)
Contraception		
No protection	15	18
Intrauterine device	15	18
Birth control pills	10	12
Condom	38	45
Tubal ligation	6	7
Nulligravid	5	6
Nulliparous	14	17
Multiparous	65	77
Smokers	17	20

Table 2. — *The immediate colposcopy results of women with HPV(-) ASC cases.*

Colposcopy results	Number	Percentage*
No dysplasia	62	74
Low-grade SIL (CIN 1)	19	23
High-grade SIL (CIN 2-3)	3	4
Total	84	

*Figures were rounded. CIN: cervical intraepithelial neoplasia. SIL: squamous intraepithelial lesion.

tions were considered as abnormal findings and biopsy was performed on these regions. Colposcopic biopsies were evaluated by the same pathologist who evaluated smears. Loop electrosurgical excision procedure (LEEP) was performed in patients that required further treatment.

Terminology

For Pap smear test, TBS 2001 classification was utilized. For colposcopy, Barcelona 2002 terminology was preferred. For HrHPV tests, results were given as "screening HrHPV(+)" and when detection was positive, genotyping were given as "16+, 18+ or other HrHPV(+)". Other HrHPV(+) denoted presence of other high-risk HPV DNA which was different from 16 and 18 types (i.e. 31,33,35,39,45,51,52,56,58,59,66, and 68).

For colposcopic biopsy results, novel recommended terminology The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions (LAST, 2012) was used [5, 6]. In this terminology, biopsy material was also classified similar to cytological counterparts. In LAST 2012, cervical intraepithelial neoplasia (CIN)1 and equivalent lesions in "biopsy material" was termed as "low-grade squamous intraepithelial lesion", whereas CIN2 and CIN3 were grouped as "high-grade squamous intraepithelial lesion". During evaluation of biopsy material, the authors re-evaluated pertinent cytological material once again. The age, parity, smoking habits, and the contraceptive methods of the enrolled patients were also questioned and recorded.

Retrospective review of squamous intraepithelial lesion (SIL) cases

The authors retrospectively reviewed pathology archives to search for patients with SIL that had cervical cytology which was prior to current test. They could not identify a prior cervical cytological test of cases with SIL diagnosis, in their records. P16 staining of biopsy materials were not available. All percentile data were rounded to give clarity. Statistical evaluation was made with SPSS 15.0 software.

Table 3. — *Univariate statistical analysis of demographic and gynecological parameters. All cases are diagnosed as atypical squamous cells and HrHPV(-). High-grade squamous lesions denote presence of CIN 2-3 in biopsy material; "other" include low-grade SIL and benign reactive conditions.*

Risk factor	HSIL	Other	*p* value[a]
Smoking status	3	14	0.007
Parity (Nulligravid *vs.* non-nulligravid)	3	81	0.670
IUD use	3	12	0.005
Contraception (barrier)	0	3	0.280
Age (years, median 36, range 31 to 46)			0.947[b]

[a] *p*-value is calculated by Fisher exact test. [b]Non-parametric tests.

Table 4. — *Linear regression analysis of variables in current analysis.*

Risk factors	LSIL+HSIL R2 0.113 Unstandardized coefficients (B)	*p*
Smoking status	0.001	0.994
Parity	-0.026	0.649
Contraception	-0.067	0.105
Contraception (barrier)	-0.313	0.011
Age (years, median 36, range 31 to 46)	0.006	0.659

LSIL: low-grade squamous intraepithelial lesion;
HSIL: high-grade squamous intraepithelial lesions.

Results

During a one-year period, a total of 116 patients were evaluated. Of these cases, 104 had a valid HrHPV test. Six patients did not apply for colposcopic evaluation. In two cases, colposcopy was unsatisfactory. Except for one case with bleeding, no complication was observed colposcopy and biopsy procedure. In this case simple suturing was sufficient to stop bleeding. A flowchart outlining the breakdown of cases are given in Figure 1. Median age of participants was 36 years (range from 31 to 45). Contraception, gravida status, and other pertinent gynecological information are given in Table 1.

Colposcopic biopsy revealed LSIL in 19 cases (22%) and HSIL in three cases (4%). Sixty-two patients were classified as "no dysplasia" (Table 2. Adenocarcinoma in situ or invasive cervical cancer were not detected. Patients that were found to have LSIL on colposcopic biopsy, were scheduled to follow up in one year. LEEP was performed in three cases with HSIL. Histopathological assessment identified a high grade SIL in all three cases. In all cases, surgical margins were free of lesion.

Statistical analysis showed that, of all gynecological and demographic parameters in current group, smoking and ıntrauterine device use (IUD) use significantly associated with harboring HSIL in HrHPV(-)ASCUS cases (Tables 3 and 4).

Discussion

The cervical cancer screening community had recently a great interest in power of HPV testing for cervical cancer screening. Probably as result of this growing confidence on HPV testing, rationale for establishing clinical guidelines shifted on HPV tests [7]. Although test performance of HrHPV testing has been studied extensively, FN fraction of HrHPV tests have been evaluated in limited number of studies [8-12]. Detection rate of HSIL in ASC-US/HrHPV (-) cases has been reported to range from 0.06 to 7%. Rate is higher in studies that utilize "immediate colposcopy" rather than follow-up. Lonky *et al.* utilized hybrid capture 2 (HCII) and performed colposcopy in all ASC-US cases. In this study, (HCII test was negative in 25% of cases that turned out to harbor high-grade SIL [9]. In the same study, the authors also compared biopsy diagnoses and HCII results in cases with ASC-US (subgroups), they showed that 6% of ASC-US/HCII(-) cases had HSIL on biopsies. Eltoum *et al.* performed a review of ASC-US cases with negative HPV DNA(-) tests, 7% of cases showed CIN2+ in their cervical tissue biopsies [8]. Based on these observations, up to 10% of HrHPV(-) ASC-US cases might be expected to have HSIL at time of application.

Risk at baseline screening (date on which patient applied for screening) is the risk of having HSIL on concomitant or immediate colposcopy. Studies that assess baseline or imminent risk of HSIL in cases with HrHPV negative ASC-US cases, are limited to few, since these patients have been usually recommended to follow-up in three to five years. Castle *et al.* analyzed HrHPV negative cervical precancers in the ASC-US-LSIL triage group [13]. In this analysis, there were 33 (6% of total) CIN3 cases with baseline HrHPV(-). Of these cases, 75.8% had ASC-US diagnosis in their referral Pap test.

Main limitation of any study that evaluates the outcome of HrHPV negative ASC-US cases, is the lack of sufficient follow-up information on all cases. Most of information about such cases is available through health maintenance organization (HMO) based studies [9-11,14]. Katki *et al.* analyzed follow up results of a large population. They evaluated a HMO-based population in which, women with HPV negative/ASC-US results should be re-evaluated in one year [11]. Their results showed 0.6% of HPV negative ASC-US cases had high-grade lesions. Furthermore, their data showed that 0.06% of invasive cancers is in same group (HrHPV-). Although these data are based on follow-up information rather than baseline colposcopy analysis, authors regarded these data as valuable and they concluded that ASC-US Pap test smear result may imply a risk information in the absence of detectable HPV. Safaeian *et al.* evaluated 12 months' follow-up of HPV negative cases with ASC-US diagnosis [15]. In HPV negative ASC-US arm, 3.1% of cases had HSIL (CIN2 and CIN3).

In the current study, the authors searched for concurrent biopsy results rather than one-year follow-up results with cytology (and/or HPV testing). This approach may be looked at differently from what most gynecologists would prefer. They would perform a repeat Pap or HPV-only at one-year for an ASC-US. Using guidelines intervals of three to five years, the rate of transformation from HSIL to invasive cancer is relatively low. Even women with Pap-/HPV- testing have a five-year risk of about 0.08% HSIL risk [14]. Regression rates of HSIL is out of the scope of the present study, since the authors would like to focus on immediate risk of having HSIL rather than regression rates.

FN HPV tests have been reported to occur. Zuna *et al.* utilized both hybride capture and PCR and found out that 2-11% of LSIL cases were HPV negative [16]. In the same study, the authors showed that 12-32% of women with LSIL that had HPV negative tests, tested positive at their six-month follow-up visit. When cytological diagnosis is not concerned, overall FN results in HrHPV testing are reported up to be 17% (range 0-17%) of the screened population [17-19].

A limited number of studies are present in medical literature that specifically seek FN rates of HPV tests. In ASCUS-LSIL Triage Study (ALTS study), FN HPV rate was 4.1% to 15% for HSIL (CIN2-3). When all CIN lesions were considered together, this rate was about 36.6 %. In a recent study, 3.1% of women with HSIL had negative HPV tests with HCII [20].

Implied factors in FN results of HrHPV test are HPV genotypes in tests, short term fluctuations of HPV DNA, viral clearance before histological resolution of a specific lesion, histological confirmation of biopsy materials, collection and sampling techniques, and type of HPV tests [18, 19]. Size of lesion might also affect the HPV test with sampling and detection errors [8, 21]. Lonky *et al.* showed that all HSIL cases whose cytological material were HPV negative (with HCII test), had evidence of HrHPV DNA in biopsy material. The authors suggested that cytological sample may not represent actual HPV infection and HPV tests are apt to harbor errors related to exfoliation/sampling [9]. Insufficient cellularity, lower viral DNA load, and anatomic features of cervical os (i.e. larger os) have been linked to correspond to FN HPV tests [10-12]. In a recent study, it was noticed that women with smaller lesions or nonsatisfactory colposcopy have a higher rate of HCII HPV negative test results [20].

In addition to test related factors for FN results, another major issue is the correct histological assessment of tissue biopsies. In the current study, the authors are confident with histological diagnosis, since all patients with high-grade lesions underwent loop electrosurgical procedure and pathological examination confirmed colposcopic biopsy diagnosis.

The reproducibility of cervical cytology and corresponding biopsy diagnoses is critical for evaluating the effectiveness of a screening/detection method. Biopsy reports along with colposcopy are regarded as "gold standard" to measure the impact of new screening and vaccine tech-

nologies. However there are inherent issues in the interpretation of cervical biopsy materials. First, the histological features of cervical pre-neoplastic lesions are observed as a spectrum of changes over timeline. Current evidence showed that CIN1 lesion and koilocytic change are related to the lytic cycle of HPV infection. On the contrary to CIN1, CIN3 lesions are reported to have viral integration to epithelial cell DNA and CIN3 lesions have capacity to progress to invasive cervical carcinoma. Whereas viral integration and risk for progressing invasive carcinoma are well-established for CIN3 and CIN1, the meaning of CIN2 diagnosis is less clear. In the present study, the authors preferred the use of novel terminology LAST as advised [6]. In this terminology, cervical squamous lesions are termed as high-grade (CIN2-3) and low grade (CIN1) SIL. This novel terminology also assisted them to avoid the CIN2 category in biopsy results. In recent multinational analysis it was reported that diagnosing a squamous lesion as CIN2 is much less reproducible and agreement among different pathologists on same lesion is poor [22]. In addition to reproducibility issues, the term CIN2 has equivocal meanings for neoplastic capacity and virus-host interactions.

PCR based HrHPV detection tests were utilized in current assessment. A variety of detection methods for HPV has been implemented in clinical and laboratory practice. These tests usually are based on three methodologies, i.e. nucleic acid hybridization (direct methods), signal amplification, and nucleic acid amplification (target amplification) assays. Of these methods, nucleic acid hybridization assays have highest specificity and are being considered as gold standard (Southern blot hybridization). However, nucleic acid hybridization methods are labor-intensive and relatively expensive. In addition, these assays require specific samples, so that DNA should be preserved as not to be degraded. Signal amplification assay methods are relatively cheaper, and easy to perform. The most widespread utilized tests (HCII) are in this group. Nucleic acid amplification assays utilize a well- known procedure of PCR. They are also referred to as PCR- based tests. This group of tests are relatively new in market and their prices are competitive. Among all methodologies, this group today is the most demanded one, probably due to its competitive prices, highest specificity, and easy to perform in automatic processors that utilize common cervical sample containers. Target amplification methods are also suitable for high volume of tests.

Actually how much risk is being taken when deferring a patient (HPV negative ASC-US case) for three years? Cumulative risk to develop a high-grade lesion over the years are well-studied in articles that evaluate five-year risks of CIN3+ and cervical cancer in HrHPV(-)/ASC-US cases [14]. Authors showed that over a five-year period, CIN3+ cumulative risk was ten-fold (0.043 at baseline and 0.43 at five years). Same risk is 13-times more for CIN2 (0.08 at baseline and 1.1 at five years). At three years, these risks are ten-fold for CIN2 (0.0 at baseline and 0.75 at three years) and six-fold for CIN3+ (0.043 at baseline and 0.29 at three years). Adding ten-fold risk (CIN2) and six-fold risk (CIN3) to the probability of having baseline HSIL in this group would yield an estimate for how much risk is taken.

Well-organized clinical trials and large studies stated that HPV testing is sufficient for preventing CIN3/AIS, however in real world it is not clear whether HPV testing prevent cervical carcinoma [18]. Even in well-organized screening programs, which include HPV testing and Pap tests together, cervical carcinoma was seen in about 3/100,000 participants [11]. This risk is not different with the population that was screened with Pap smear alone. When risk of cancer is concerned, performance of HPV based triage of cases with ASC-US are still subject to debate in spite of results of controlled clinical trials. A population-based study in routine clinical practice showed that of 6,496 women with baseline HPV negative/ASC-US, 40 patients (5%) had high-grade lesions (CIN2, CIN3 and AIS). Interestingly two (2%) women were reported to have invasive carcinoma [18]. The present data showed an absolute 4% cases in this group have high-grade SIL at the time of HPV testing. Deferring these patients for another three years may increase the risk of invasive cervical cancer.

In the present study, the authors also noticed that IUD use and smoking status were significantly correlated with presence of HSIL in HPV negative/ASC-US patients. Whereas smoking is well-known predisposing factor for cervical dysplasia, IUD use is controversial [23-25]. Data in the current study is limited to draw conclusions. However, to minimize risk of invasive cancer development, the authors believe that smokers should be notified about the regular follow-ups.

Triage of women with ASC-US diagnosis in cytological material and negative HPV testing are similar in different guidelines. Currently, the American Congress of Obstetricians and Gynecologists(ACOG) guidelines recommend routine screening for such cases as indicated for their age. However, ACOG also notices that the strength of this recommendation is level B, which denotes that recommendation is based on limited and inconsistent scientific evidences [26]. In American Society for Colposcopy and Cervical Pathology (ASCCP)'s Updated Consensus Guidelines for Managing Abnormal Cervical Cancer Screening Tests and Cancer Precursors, co-test should be repeated in women with ASC-US diagnosis and negative HPV tests [4].

FN risk of HrHPV in ASC cases has been reported as minimally as 0.4% in some studies. In epidemiologist's point of view, this rate might be regarded as "negligible" whereas in media and newspaper point of view it may not, especially when a woman with previously diagnosed as HrHPV(-) twice and have cervical cancer (Ortega Bob."False-negative results found in HPV testing" January14, 2013, The Arizona Republic,12:36 a.m. EST January14,2013). Based on the present results and similar data in literature, a small percentage (less than 5%) of ASC-US HPV negative cases are expected to have HSIL in follow-up periods less than six months.

Zhou *et al.* recently reviewed prior HrHPV test results of patients with invasive cervical cancer in a retrospective multicenter study [27]. Authors showed that 9% of patients with invasive cervical cancer had HrHPV negative test in "one year" prior to cancer diagnosis. Whereas negative HPV test rates were 23% one to three years prior to cancer diagnosis, this rate was 25% three to five years prior to diagnosis. Based on similar observations and current findings, follow-up which is not more than one year, preferably in six months, would be a reasonable approach in patients with ASCUS/HPV(-).

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