Original Article
Atypical squamous cells of undetermined significance in patients with HPV positive DNA testing and correlation with disease progression by age group: an institutional experience

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Abstract: Atypical squamous cells of undetermined significance (ASC-US) is a broad diagnostic category that could be attributed to human papillomavirus infection (HPV), malignant neoplasia and reactive conditions. We evaluated our institutional experience with ASC-US in women who are positive for high risk HPV (HRHPV+) by the Digene hybrid capture method from 2005-2009 to identify the risk of progression to squamous intraepithelial lesion (SIL) and cervical intraepithelial neoplasia (CIN) in association with age. We reviewed cytologic and follow-up surgical pathology reports for all specimens available. Progression was defined as a diagnosis of at least CINI on follow-up biopsy or resection or SIL on cytology. We identified 2613 cases and follow-up was available in 1839 (70.4%). Of these 74.2% had just one follow-up, 16.2% had a total of 2 follow-ups, 5.3% had a total of 3 follow-ups, and the remaining had as many as 6 follow-ups. Among the 1839 patients, 69.4% were age 30 or younger, 16.0% were between 31 to 40, 9.0% were between 41 to 50, and 5.6% were 51 or older. Among these, 25-30% progressed to dysplasia. The risk of progression varied by age (p=0.04) and was lowest among women between the ages of 41-50. Our findings highlight the importance of continued cytologic follow-up in women with HRHPV+ ASC-US in order to detect progression of disease, although the risk of progression is age dependent.

Keywords: ASC-US, Pap smear, HPV, Digene hybrid capture, cervical cytology

Introduction

Cervical cancer is one of the most common malignancies in women worldwide. Human papillomavirus infection (HPV) has been implicated as the major etiologic agent for the majority of cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma of the cervix [1, 2]. The rate of infection in young females after the onset of sexual activity is high. However, HPV infection is usually a self limited process and does not lead to significant epithelial lesions or cancer in most cases. Nevertheless, persistent infection or multiple infections are associated with an increased risk of developing a cervical precursor or an invasive epithelial lesion [1-4]. Routine screening cytology (Pap tests) has played an essential role in the early detection of these various lesions, leading to a significant decrease in cervical cancer mortality [5].

In screening cytology, an important morphologic problem is the presence of what has been designated as "atypical squamous cells (ASC)". The Bethesda system for reporting cervical cytology defines atypical squamous cells (ASC) as a diagnostic category in which the epithelial cells have changes suggestive of squamous intraepithelial lesions, but are insufficient for a definitive diagnosis of preneoplasia because of cell quantity or qualitative aspects. It also recommends all ASC diagnosis should be qualified as "Of undetermined significance" (ASC-US) or "cannot exclude high grade intraepithelial lesion (ASC-H).

Atypical squamous cells of undetermined significance (ASC-US) is a broad diagnostic category in gynecologic cytology that comprises several etiologic processes including HPV infection, malignant neoplasia and reactive conditions [5].

HPV DNA molecular testing in conjunction with cytology has been approved by the US Food and Drug Administration to be used as a cervical cancer screening tool in women older than 30 years of age, in particular for the triage of women with a diagnosis of ASC-US. There is accumulating literature suggesting that the significance of an ASC-US diagnosis may be age dependent, and that risk of progression to dysplasia in women under age 20 is increased while it is decreased in women over 50 [6, 7]. The aim of this study was to evaluate our experience in a large cytology screening clinical service with ASC-US diagnoses in patients harboring high risk HPV positivity (HRHPV+), and demonstrates the significance of the risk of progression in association with age along with the cytologic and histologic follow up findings.

Methods

Patients

This study was approved by the Institutional Review Board at Mayo Clinic. We retrospectively queried our pathology database (SNOMED) for all patients with a diagnosis of ASC-US and HPV positive results from 2005 to 2009. Criteria for inclusion included a diagnosis of ASC-US followed by a positive HPV result by molecular diagnosis. We reviewed cytologic and follow-up surgical pathology reports for all specimens available, including pap smears, biopsies and resections, and documented number of follow-ups.

Definition of follow-up

Follow up was defined as patients having colposcopy with either cervical biopsy with/without endocervical sampling, LEEP, cone biopsy, or repeat cytology. Repeat cytology after colposcopy has shown to increase sensitivity for detecting dysplasia; therefore, both forms of follow up were included [8, 9].

Definition of progression

Progression was defined as a diagnosis of at least low-grade squamous intraepithelial lesion (LSIL) on follow-up cytology, or at least CIN1 on biopsy or resection after 60 days from the diagnosis of ASC-US, HRHPV+. We considered a positive cytologic progression when the follow up cytologic diagnosis rendered was one of the following: ASC-H, LSIL, cannot exclude high grade SIL, or high grade SIL, squamous cell carcinoma or adenocarcinoma. Although "progression" from ASC-US to ASC-H is subject to interobserver variability, it was considered progression due to the cytologic suspicion of a more advanced lesion. Positive histologic progression was colposcopic biopsy showing mild (CIN1), moderate (CIN-2) or severe squamous dysplasia (CIN3).

Pathology

Cytologic specimens were prepared using Papanicolaou-stained ThinPrep® (Hologic, Inc., Bedford, MA) slides for diagnosis by a cytopathologist. Criteria for ASC-US and positive cytology were used in accordance with the 2001 Bethesda system classification for reporting cervical cytology [10]. In brief, ASC-US criteria included nuclei 2.5-3 x larger than an intermediate squamous cell nucleus, increased nuclear-cytoplasmic ratio, minimal hyperchromasia and chromatin irregularities, and rarely "atypical parakeratosis" [5].

HPV molecular testing

HPV results were obtained using the Digene hybrid capture method which was performed according to the manufacturer's recommendations using the high-risk probes with the positive and negative control samples run in triplicate and result validation using HCII software, version 2.0. The high-risk HPV panel consisted of 13 serotypes of HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Cytologic diagnoses were provided without knowledge of HPV status.

Statistics

Time to progression was determined as the number of days from baseline (initial positive HPV molecular test) to first progression diagnosis among those with at least 1 follow-up. Time to progression was described using ranges, interquartile ranges, and medians, and was illustrated using Kaplan-Meier curves. Risk of pro-

gression was compared between age groups (<30, 31-40, 41-50, and >51) with the log-rank test. A p-value less than 0.05 was considered statistically significant for the overall comparison by age. For the six pairwise comparisons between the four age groups, p-values less than 0.008 were considered statistically significant, using a Bonferroni correction for multiple comparisons. SAS version 9 (Cary, NC) was used for statistical analysis. Patients with no follow-up were excluded from the time-to- progression analysis.

Results

Relative frequencies of various cytology diagnostic categories

The Mayo Clinic cytology lab processed 123,763 liquid based cervical cytology specimens using the Thin Prep method from January 2005 - December 2009. Samples included both in-house and referral cases. The average ASC-US/SIL ratio for the Mayo laboratory was 1.27 and High Risk HPV positivity rate/ASC-US was 0.37. Our high grade squamous intraepithelial lesion (HSIL) rate falls between 0.4-0.5% (between 25th-50th percentiles nationally compared to CAP benchmarks) [11, 12].

Frequency of ASC-US and HRHPV positivity

During the 48 month study, a total of 6986 (5.6% of total cytology specimens) cases were diagnosed as ASC-US using the criteria described above. Of these, 2613 cases (2.0% of total cytology specimens) were ASC-US, HRHPV+ (HRHPV+ rate = 37.4%). The age distribution of these patients was: 1849 (70.8%) patients were <30 years old, 397 (15.2%) patients were 31-40 years old, 219 (8.4%) were 41-50 years old, and 148 (5.7%) patients were older than 51years of age. These results are summarized in **Table 1.**

Table 1. Age distribution of all patients diagnosed with ASC-US and HPV+.

Age group	Number of patients (%)
<30	1849 (70.8%)
31-40	397 (15.2%)
41-50	219 (8.4%)
>51	148 (5.7%)
Total	2613

Table 2. Number of follow ups and distribution of all patients with ASC-US HPV+.

Follow ups (number)	Patients with ASC-US HPV+ (%)
0	774 (29.6%)
1	1366 (52.3%)
2	298 (11.4%)
3	98 (3.8%)
4+	77 (2.9%)
Total	2613

Follow-up time in ASC-US, HRHPV + patients

A total of 1839 patients (70.4%) had follow-up, while 774 patients (29.6%) had no follow-up. The number of days from baseline diagnosis of ASC-US to the first follow-up ranged from 1-1711 days with a median of 203 days (IQR 49-362). The number of days of total follow up ranged from 1-1905 days with a median of 263 days (IQR 132-474). Interestingly, 346 patients (19%) progressed before 60 days. The number of follow-up was distributed as follows: one follow-up (n=1366; 52.3%), two follow-ups (n=298; 11.4%), three follow-ups (n=98; 3.8%), four or more follow-ups (n=77; 2.9%). The results are summarized in **Table 2.**

Cytologic or pathologic progression is frequent in patients with ASC-US, HRHPV+

A total of 717 (39%), patients had a diagnosis of reactive changes; the number and percentage of reactive changes by age group respectively was: 479 (26%) in the group <30 years old; 119 (6.4%) in the group 30 to 40 years old; 84 (4.6%) in the group 41 to 50 years old, and 35 (1.9%) in the group older than 50 years old. When focusing on dysplasia, a total of 697 patients (37.9%) had a diagnosis of CIN 1. 497 (27%) in the group <30 years old; 113 (6.1%) in the group 30 to 40 years old; 53 (2.9%) in the group 41 to 50 years old; and 34 (1.8%) in the group older than 50 years old. A total of 115 patients (6.3%) had a diagnosis of CIN2 distributed as follows: 87 (4.7%) in the group <30 years old; 16 (0.9%) in the group 30 to 40 years old; 7 (0.4%) in the group 41 to 50 years old; 5 (0.2%) in the group older than 50 years old. A total of 51 patients (2.8%) had a diagnosis of CIN3; 34 (1.8%) in the group <30 years old; 10 (0.5%) in the group 30 to 40 years; 2 (0.1%) in the group 41 to 50 years old; 5 (0.3%) in the

Table 3. Distribution of diagnostic categories by age group showing the higher lesion the patient had on follow up cytology or tissue specimen

Table of Diagnoses by Age Category

	< 30y	30 to 40y	41 to 50y	> 50y	Total
Reactive	479	119	84	35	717
	26.0%	6.4%	4.6%	1.9%	39.0%
CIN1 (mild dysplasia)	497	113	53	34	697
	27.0%	6.1%	2.9%	1.8%	37.9%
CIN2 (moderate/High grade dysplasia)	87	16	7	5	115
	4.7%	0.9%	0.4%	0.2%	6.3%
CIN3 (severe/high grade dysplasia)	34	10	2	5	51
	1.8%	0.5%	0.1%	0.3%	2.8%
Glandular atypia	4	1	0	0	5
	0.2%	0.1%	0.0%	0.0%	0.3%
Adenocarcinoma (invasive)	1	0	0	0	1
	0.1%	0.0%	0.0%	0.0%	0.1%
ASC-US HPV-	63	6	2	3	74
	3.4%	0.3%	0.1%	0.1%	4.0%
ASC-US HPV+	84	18	14	13	129
	4.6%	1.0%	0.8%	0.8%	7.0%
CIN1H (mild dysplasia & cannot exclude high grade dysplasia)	8	5	3	1	17
	0.4%	0.2%	0.1%	0.1%	0.9%
ASC-US & no HPV done	7	0	1	3	11
	0.4%	0.0%	0.1%	0.1%	0.6%
ASCH (cannot exclude high grade dysplasia)	12	7	0	3	22
	0.7%	0.4%	0.0%	0.1%	1.1%
Total	1276	295	166	102	1839
	69.4%	16.0%	9.0%	5.6%	100.00
No Follow-up (not included in percentages or total)	573	102	53	46	774

group older than 50 years old. Invasive adenocarcinoma was present in 1 patient (0.1%) younger than 30 years of age (see **Table 3**). In summary, on cytologic or pathologic follow-up, 49% of patients progressed and 51% did not progress (**Figure 1**). Most of the significant lesions (i.e.CIN2-3) were found primarily in women younger than 40. In addition, 4% of patients had a negative HPV test on follow-up and 7% of the patients continued having a positive HPV test, but did not show progression.

Risk of progression in ASC-US, HRHPV+ patients is age dependent

When stratifying risk of progression by age groups, Kaplan-Meier curves demonstrated statistically significant differences (p=0.04) (**Figure 2**). Pairwise comparisons revealed a lower risk of progression for women in their 40's as compared to younger women (p=0.006). The risk of progression between the remaining three age groups did not differ significantly.

Discussion

The Bethesda system recommends the use of ASC category when the abnormalities seen in squamous cells are too pronounced to be attributed solely to reactive changes, but the changes are not enough to be categorize as SIL [5]. The ASC category should be further qualified as ASC-US, implying that the changes seen are suggestive of low grade intraepithelial neoplasia (LSIL) or intraepithelial neoplasia of undetermined grade. If a high grade squamous intraepithelial lesion (HSIL) cannot be excluded, the term used should be atypical squamous cell cannot exclude HSIL (ASC-H) [5].

The possibility to interpret epithelial changes as ASC-US is recognition of the limitation of this diagnostic method and provides opportunity to express uncertainty to warrant patient follow-up. ASC-US is reported to be a poorly reproducible diagnosis; in fact, certain diagnostic criteria, such as nuclear size, have been proven to be
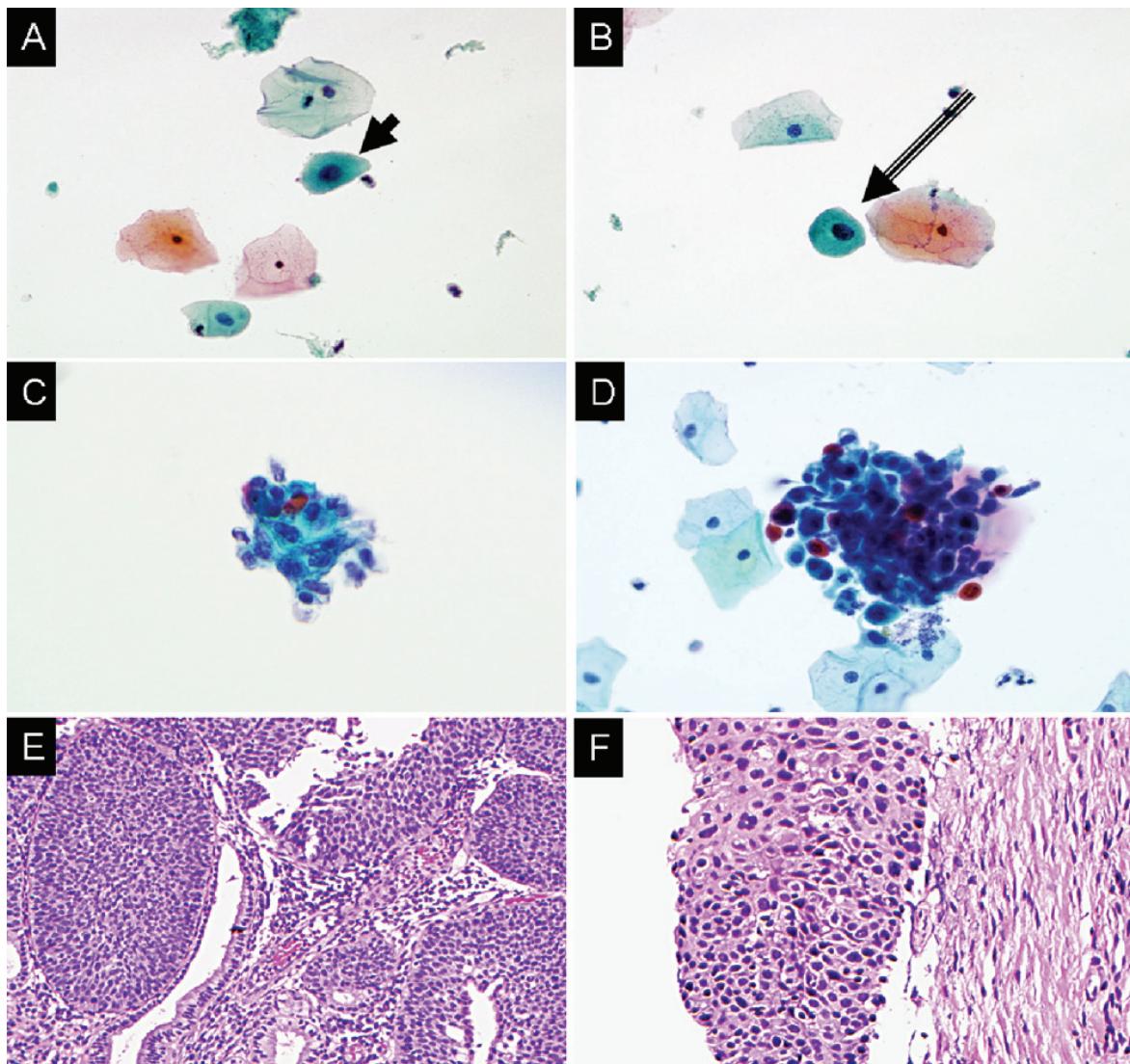
Figure 1. Cytologic and histologic features HRHPV+ lesions. The photomicrographs in Figure 1 shows two different patients as they progressed from ASC-US to severe dysplasia. ASC-US is characterized by nuclear enlargement 2 times the size of an intermediate cell nucleus (short arrow) (A) and ill-defined perinuclear halos (long arrow) (B). Both cases were HRHPV+. Progression to high grade squamous intraepithelial lesion is illustrated with nuclear pleomorphism and hyperchromasia, and a high nuclear to cytoplasmic ratio (C, D). Cervical intraepithelial neoplasia (CIN3) is confirmed with colposcopic biopsy (E, F).

nearly irreproducible [13-15]. Other features that pose challenges on this diagnostic category are physiologic changes that occur with age. Perimenopausal and postmenopausal patients exhibit cytologic features of atrophic changes such as nuclear hyperchromasia, pleomorphism, and perinuclear halos. These findings were often diagnosed as ASC-US, resulting in overdiagnosis of physiologic changes as abnormal [16, 17].

The main differential diagnosis of ASC-US are benign/reactive changes secondary to different stimuli and HPV cytopathic effect/SIL [5]. Nevertheless, ASC-US is an important diagnostic category. If this category was eliminated, by downgrading these changes as reactive or overinterpreting these lesions as squamous intraepithelial neoplasia, it would decrease the accuracy of the Papanicolaou test as well as decrease its sensitivity [18].
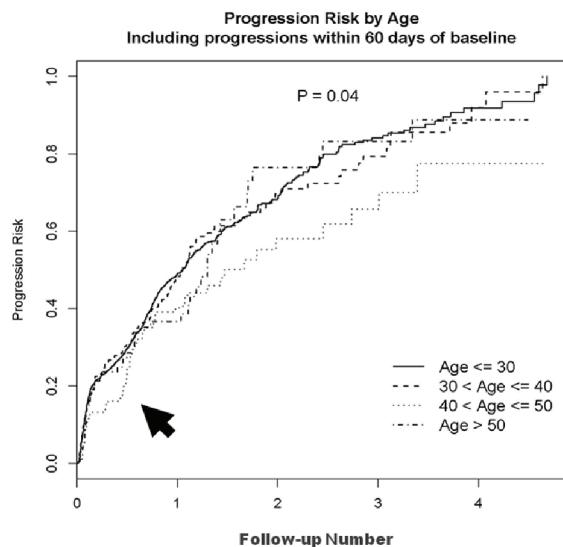
Figure 2. Risk of progression by age group. Kaplan-Meyer curve showing the risk of progression. The dip at the beginning of the curve (dark arrow) in the 41-50 age group shows that, ASC-US at the first follow up, has a lower risk of finding a more significant lesion. The risk of progression between the remaining three age groups did not differ significantly.

Earlier attempts to stratify the risk of patients with a diagnosis of ASC-US who progressed to intraepithelial neoplasia based on morphologic criteria solely showed that the risk to progression appeared to vary according to age groups. Pediatric and adolescent patients were at high risk to progress to SIL/CIN [19, 20] while women older than 50 years old had a lower frequency to progression [21].

Infection by high grade HPV has been recognized as a major etiologic factor in cervical cancer [1, 2]. Cytology triage with high risk HPV testing has proven to be more sensitive and specific than conventional cytology triage. HPV testing is an important ancillary diagnostic tool in distinguishing which women are at risk to progress to SIL, decreasing the number of colposcopy referrals and follow-up tests [22, 23].

The reported prevalence of HPV infection in the literature varies widely. The presence of HRHPV+ as well as ASC-US HRHPV+ has been shown to be inversely proportional to age [24].

In adolescents with ASC-US, Boardman et al demonstrated an HPV DNA rate of 80%. Musa

et al showed that women younger than 30 years old carrying a diagnosis of ASC-US were significantly more likely to be HRHPV+ [25]. Ko et al reported that in women 20 years or younger, the HRHPV rate among women with ASC-US was up to 50%, compared to only 15% in patients over 50 [11]. Johnston et al reported that 43.8% of women with ASC-US age 10 to 39 years were positive for HPV DNA compared with 20.5% of women age older than 40 years [21]. These frequencies are somewhat similar to the ones in our current study. When focusing on progression to intraepithelial neoplasia, in our cohort 49% of the patients progressed and 51% did not progress. Women in the 41-50 age range had a lower risk of progression than younger women, which is in agreement with previous studies [7, 16, 19, 21, 23, 24, 26]. It is important to mention that our patient population is considered of normal risk for HPV and CIN compared to other populations in the United States.

The significance of HRHPV testing in patients older than 50 years of age may be of special interest, given the relative low frequency of patients older than 50 years of age in ASCUS/HRHPV positive populations. For example, on Armah et al study of follow-up findings on patients with ASC-US and HRHPV+ showed that 3.2% of women older than 50 years of age had a follow-up of CIN2/CIN3 [27]. We report a similar rate of patients older than 50 years in our study. A total of 158 (5.7%) patients were older than 51 years of age and of these 10 patients (6.3%) showed progression to CIN2/CIN3. However, other studies have highlighted the importance of HRHPV testing in older age groups. For example, the study of Zhao et al showed that in women 50 years of age and older, a positive HRHPV test result significantly increased the likelihood of follow-up histopathologic diagnoses of CIN2/CIN3 in patients with cytologic diagnosis of HSIL, LSIL, and ASC-H Papanicolaou test results, compared with patients with negative HRHPV test results [7]. This may support the utility of HRHPV testing in women older than 50 years of age.

In our study there were approximately 20% (n=346) of women who had confirmation of a higher grade lesion either on follow up cytology or colposcopy prior to 60 days of the HRHPV+ ASC-US. It is well known that 10-20% of patients with atypical squamous findings have an asso-

ciation with CIN2/CIN3 [28]. We believe these women may have infection for a longer time and the initial cytology may have been undercalled as ASC-US, since some authors have reported that the risk of developing SIL is associated with having had HPV infection for at least 6 months [22]. The reasons why these women returned to follow-up prior to 60 days after diagnosis is unclear.

There were some limitations in our current study to be aware of. Our study is retrospective in nature and covers a limited timeframe; therefore it is not possible to know with certainty the timing of HPV infection in relation to the cytologic findings. As mentioned above, our patient cohort is considered normal risk for HPV, and the results may not be generalized to other groups, in particular higher risk populations with regards to progression risk. However, our data compares favorably with other studies, and highlights the age-dependent associations with respect to progression in ASC-US HRHPV+ patients.

In conclusion, we report our experience with the cytologic category of ASC-US and HRHPV testing in a large tertiary care center, with normal HPV risk. We confirm the age dependent association of progression risk in this population, although additional studies may be required to specify in detail the exact contribution of age to our observations.

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