RESEARCH ARTICLE

Evaluating the Existence of Small Compressed Binucleated Squamous Cells in ASC-H

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Abstract

Purpose: To evaluate the legitimacy of a diagnosis of ASC-H in 5 cases which were followed up monthly for over 2 years with both cytology and HPV testing. **Methods:** Some 5 cases out of a total of 25.0 self-sampled Pap test patients diagnosed as ASC-H provided 119 specimens over 2 years, with HPV-DNA testing perormed using a E6 primer. **Results:** Cases 1, 2 and 3 showed SIL after the ASC-H diagnosis, while cases 4 and 5 showed and maintained NILM. Cases 1, 2 and 3 were further characterized by small atypical compressed binucleated cells, in which HPV was detected by *in situ* PCR. Case 4 showed a high N/C ratio in cells in sheets with a mild increase in chromatin. Case 5 demonstrated a high N/C ratio in small cells with no increase in chromatin. **Conclusion:** The finding of a compressed binucleated cells can define the difference between degenerated endocervical columnar cells and small atypical cells suggestive of HSIL. When small compressed binucleated squamous cells are detected, there may be a chance of continuing HPV infection and undetected SIL.

Keywords: HPV- ASC-H- compressed binucleated squamous cells

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Introduction

In April of 2009, the 2001 Bethesda system was introduced and implemented in the Japanese reporting system of cytology. In addition to the established diagnostic categories, ASC (Atypical Squamous Cells) category was added (Solomon et al., 2002). Within the ASC category, an ASC-H (Atypical Squamous Cells, cannot exclude HSIL) diagnosis is especially important since it indicates that those cells are suspicious for HSIL (High-grade Squamous Intraepithelial Lesion), and that there is a high probability of detecting a CIN2 or higher lesion on cervical biopsy (Sherman et al., 2006).

The cytological criteria of ASC-H is divided into two main groups, single or few cell groups of small atypical cells and sheet like appearance. However the nature of HSIL cells are small atypical cells, and it is not rare to have difficulty differentiating between true HSIL atypical cells and benign reserve cell hyperplasia, endocervical glandular cells and other benign degenerative changes, creating a noticeable gap in diagnoses among cytotechs or institutions (Louro et al., 2013).

Our study is to evaluate the legitimacy of a diagnosis of ASC-H.

Materials and Methods

Patients

There were 25 female patients who participated in monthly mail-in Pap test using Kato self-sampling device from January 2008 to June 2012. Those specimens were processed with Liquid-based cytology and evaluated. Out of those 25 patients, 5 were diagnosed as ASC-H and preferred to have monthly cytology follow-ups over colposcopy and biopsy follow-ups. Collected number of specimens with its durations are; 34 specimens over 41 months for Case 1, 25 specimens over 31 month for Case 2, 22 specimens over 27 months for Case 3, 17 specimens over 25 months for Case 4 and 21 specimens over 25 months for Case 5. Case 1, 2 and 3 developed SIL after the original ASC-H diagnosis. Case 4 and 5 remained NILM (Negative for Intraepithelial Lesion or Malignancy) throughout the follow-up duration. No biopsy or treatments were done during this follow-up duration for all cases.

A combined method of Kato-Self sampling device and liquid based preparation techniques (Okayama et al., 2012).

Specimens were collected using Kato-Self sampling device and were prepared using an original method similar to liquid based preparation. The abnormal cell detection

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rate of this method was similar to the rate of samples collected by gynecologists. Thus, the reliability of this combined method is higher than just processing it as directly smearing on glass from the self-sampling device, which is known to have lower abnormal cell detection rate and more inadequate specimens (Okayama et al., 2012).

Pap test

The preparations were fixed in 95% ethanol and stained using the Papanicolaou method. The samples were classified according to the modified 2001 Bethesda System: Negative for malignancy (NILM); Atypical squamous cells of undetermined significance (ASC-US); low grade squamous intraepithelial lesion (LSIL); Atypical squamous cells of undetermined significance cannot exclude a high-grade lesion (ASC-H); high-grade squamous intraepithelial lesion (HSIL); and invasive carcinoma.

HPV genotyping

DNA was extracted from liquid cervical cytology specimens (100µl) using the high pure polymerase chain reaction (PCR) template preparation kit (Roche). HPV-DNA was amplified by PCR using specific primers for the HPV E6 region (Okayama K et al, 2013). A PCR reaction mixture included 1×AmpliTaq Gold® 360buffer, 2 mM MgCl2, 0.02 U/µl AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems), 1 µl DNA, and 0.5 pM primers (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 6, and 11) in a total volume of 25 µl. PCR amplification was performed using a thermal cycler with 35 cycles of denaturation at 95°C (30 sec), annealing at 60°C (30 sec), and extension at 72°C (30 sec), including an initial denaturation step of 10 min and a final extension step for 5 min. Human β-actin expression, determined using an additional PCR method, was used as an internal standard; the resulting amplicon was 262 bp.

HPV detection by in situ PCR with HPV primers

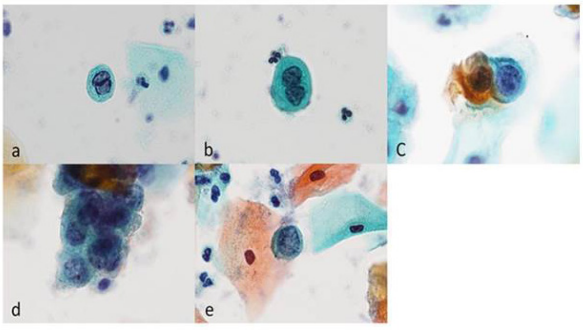

Figure 1. ASC-H Cells (Pap. Staining ×40): a) Small Atypical Compressed Binucleated Cell with High N/C Ratio and Increased Chromatin in Case 1, which Later Developed into LSIL After the Original ASC-H Diagnosis. b) Small Atypical Compressed Binucleated Cell in Case 2, which Later Developed into HSIL. c) Atypical Small Squamous Cell with High N/C Ratio and Increased Chromatin in Case 3, which Later Developed into LSIL. d) Atypical Squamous Cells in Sheet-like Structures with Somewhat High N/C Ratio in Case 4, which Remained NILM. e) Small Atypical Cell with High N/C Ratio and Mildly Increased Chromatin in Case 5, which Remained NILM.

The protocol for *in situ* PCR was based on a method devised by us our team (Okayama et al., 2010a). After decolorizing a Pap smear specimen, endogenous peroxides were removed and proteolysis was performed with 0.01% trypsin (SIGMA). The *in situ* PCR reaction mixture included 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 3.75 mM MgCl2, 0.045 U/ul Ex Taq DNA polymerase (TaKaRa), 0.2 mM digoxigenin labeling mix (Roche), and 1 pM HPV primers (Okayama et al., 2013). Specimens were enclosed within gaskets (TaKaRa), the *in situ* PCR reaction mixture was placed into the area surrounded by the gasket, and a plastic film was placed over the gasket to prevent evaporation. Slides were initially denatured for 10 minutes at 93°C, then continued onto 30 rounds of a thermal cycler, which consists of denaturation at 93°C (1 min), annealing at 60°C (1 min), and extension at 72°C (1 min). DIG incorporated into PCR amplicons was detected by an immunoperoxidase assay using anti-DIG antibody reacted for 1 hour at room temperature. The positive control was the HPV16-positive human cervical cancer cell line SiHa (ATCC®HTB-35) and the negative control was the human promyelocytic leukemia cell line HL60.

Results

Cellular criteria and HPV infection on cases 1, 2 and 3 which developed SIL after the original ASC-H diagnosis (Table 1).

Case 1: Detected ASC-H in June 2009, followed up for 41 months. In August 2009, she was diagnosed LSIL for 3 months then remained NILM after that. HPV (51) infection was detected for 5 month, from June 2009 to November 2009. This shows a correlation between LSIL cellular changes and HPV (51.0) infection. Figure 1 shows the ASC-H cells in June 2009. Small atypical cells with high N/C ratio and high chromatin. Note the presence of not only mononuclear cells but also small binucleated squamous cells. HPV (51) was detected by *in situ* PCR in small binucleated squamous cells (Figure 2).

Case 2: Detected ASC-H in April 2010, followed up 31 months and remained HPV positive (16) throughout. Subsequent LSIL diagnosis, then another 4 months of ASC-H, then finally diagnosed as HSIL. Figure 2 shows the ASC-H cells of case 2. Basal cell type atypical cells shows thick cytoplasm, which indicates metaplastic cell origin, and also shows high N/C ratio and increased coarse chromatin. Also some small compressed binucleated squamous cells were seen. HPV (16) was detected by *in situ* PCR in these cells.

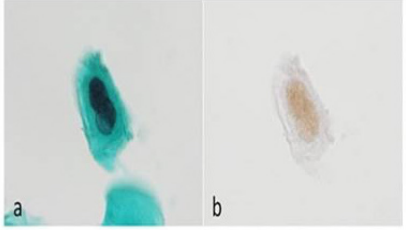

Figure 2. Small Atypical Compressed Binucleated Cell in Case 1: a) Pap. Staining (×40), b) Positive Nuclear Staining by *in situ* PCR with HPV 51 Primers (×40).

Table 1. Cellular Criteria and HPV Infection on Cases 1, 2 and 3 which Developed SIL After the Original ASC-H Diagnosis

Year	Month	Case 1	Case 2	Case 3			
		Cytology	HPV types	Cytology	HPV types	Cytology	HPV types
2008	Feb.	NILM	N				
	Mar.	ASC-US	56				
	Apr.	NILM	N	LSIL	16,39,58,68		
	May	NILM	N	NT	NT		
	June	NILM	56	NILM	16,39,58		
	July	NILM	31,56	LSIL	16,39,58,68		
	Aug.	NILM	N	NT	NT		
	Sept.	NT	NT	LSIL	16.39		
	Oct.	NT	NT	ASC-US	16,39,68		
	Nov.	NILM	N	LSIL	16,39,68		
	Dec.	NILM	N	NT	NT		
2009	Jan.	NILM	N	ASC-US	16.0		
	Feb.	NT	NT	ASC-US	16,39		
	Mar.	NILM	56	ASC-US	16,39		
	Apr.	NILM	56	LSIL	16,39		
	May	NILM	N	ASC-US	16,39		
	June	ASC-H	51	NT	NT		
	July	ASC-US	51	NILM	16,39		
	Aug.	NT	NT	NILM	16,39		
	Sept.	LSIL	51	NILM	16,39		
	Oct.	LSIL	51	NT	NT		
	Nov.	LSIL	51	NILM	N	NILM	N
	Dec.	NILM	N	NILM	16,39	NILM	N
2010	Jan.	NILM	52	NILM	16,39	NILM	N
	Feb.	NT	NT	NILM	16	NILM	N
	Mar.	ASC-US	N	NILM	16,39	NILM	N
	Apr.	NILM	52	ASC-H	16	NT	
	May	ASC-US	N	LSIL	16,39	NILM	N
	June	NILM	52	ASC-H	16	NILM	16,31,45,52,58
	July	NT	NT	NT	NT	NILM	16,31,45,52,58
	Aug.	NILM	N	ASC-H	16,39	NILM	16,31,45,52
	Sept.	NILM	N	ASC-H	16,39	NILM	16,31,45,52,58
	Oct.	NILM	N	HSIL	16,39	NT	NT
	Nov.	NILM	N			NILM	16,31,45,52
	Dec.	NT	NT			NILM	16,31,45,52
2011	Jan.	NILM	N			NILM	16,31,45,52
	Feb.	NILM	N			NT	NT
	Mar.	NILM	N			NILM	31,39,45,52
	Apr.	NILM	N			ASC-US	31,39,45,52
	May	NILM	52			NILM	31,39,45,52,58
	June	NILM	52			NT	NT
	July					ASC-H	31,39,45,52
	Aug.					NILM	31,39,45,52
	Sept.					ASC-US	31,39,45,52,58
	Oct.					NILM	31,39,45,52
	Nov.					NT	NT
	Dec.					NILM	31,39,45,52
2012	Jan.					LSIL	31,39,45,52

Abbreviation, NT, not tested; N, HPV negative

Table 2. Cellular Criteria and HPV Infection on Cases 4 and 5 which Remained NILM After the Original ASC-H Diagnosis

Year	Month	Case 4	Case 5		
		cytology	HPV types	cytology	HPV types
2010	May	NILM	68	NILM	16
	June	NILM	52,68	NT	NT
	July	ASC-H	52,68	NILM	16,45,56,68
	Aug.	NT	NT	NILM	45,68
	Sep.	NT	NT	NILM	45,56,68
	Oct.	NILM	52,68	NILM	45,56,68
	Nov.	NILM	52,68	ASC-H	45,56,68
	Dec.	NILM	52,68	NT	NT
2011	Jan.	NILM	52,68	NILM	45
	Feb.	NT	NT	NT	NT
	Mar.	NILM	52,68	NILM	45,52
	Apr.	NILM	52,68	NILM	N
	May	NILM	68	NILM	N
	June	NT	NT	NILM	N
	July	NILM	52,68	NILM	N
	Aug.	NILM	52,68	NILM	N
	Sep.	NILM	52,68	NILM	N
	Oct.	NT	NT	NILM	45
	Nov.	NILM	52,68	NILM	52
	Dec.	NILM	N	NILM	52
2012	Jan.	NT	NT	NILM	52
	Feb.	NT	NT	NT	NT
	Mar.	NILM	N	NILM	N
	Apr.	NT	NT	NILM	N
	May	NILM	N	NILM	N

Abbreviation, NT, not tested; N, HPV negative

Case 3: Detected ASC-H in July 2011, during the 17month, the patient remained HPV positive (31, 39, 45, 52), then developed to LSIL. Figure 3 shows the ASC-H cells of this patient. Small squamous cells do not show thickened cytoplasm but shows very high N/C ratio and finely distributed increased chromatin. Also some small compressed binucleated squamous cells were seen. HPV was detected by *in situ* PCR in these cells.

Cellular criteria and HPV infection on cases 4 and 5 which remained NILM after the original ASC-H diagnosis (Table 2).

Case 4: Detected ASC-H in July 2010, followed up 25 months, remained HPV positive (52 and 68). She remained NILM after the one time ASC-H diagnosis. Figure 4 shows the ASC-H cells. N/C ratio is somewhat high but appears in sheet-like structures. No increased chromatin and nucleoli were seen.

Case 5: Detected ASC-H in November 2010, followed up 25 months, remained HPV positive (45 and 68). She also remained NILM after the one time ASC-H diagnosis. Figure 5 shows scattered small atypical cells with high N/C ratio, but the level of chromatin increase was mild. Also, no atypical squamous cells that indicate mild dysplasia or HPV infections.

Discussion

CIN2 detection rate after ASC-H diagnosis is 11-79 % and it is more reliable rate than ASC-US (Simsir et al., 2006; Louro et al., 2003; Alli and Ali, 2003; Selvaggi, 2003; Quddus et al., 2001; Sherman et al., 2001; Schoolland et al., 2001; Sheils et al., 1997). However, ASC-H criteria that is explained in the 2001 Bethesda system is not as detailed, so the diagnosis of ASC-H relies on pathologists and cytotechnologists' skill levels. The fact that detection rates of CIN2 differ from institution to institution also indicates this problem. Cellular criteria that is listed in the 2001 Bethesda system are: tissue fragments/disorganized groups of hyperchromatic cells, atypical immature squamous metaplasia, and atypical mature squamous metaplasia, small atypical cells with a high nuclear/cytoplasmic ratio, atypical repair, and atrophic atypia. Among these criteria, atypical squamous metaplasia and tissue fragments/disorganized groups of hyperchromatic cells (Selvaggi, 2003) are the frequently used ones. Quddus (2001) reports that from ASC-H using those criteria lead to find HSIL in 44.2% of these ASC-H specimens. It is clear that re-defining the cytological criteria of ASC-H is urged, but we would like to suggest

a new criteria, small compressed binucleated squamous cells.

In our parallel study of cytology and HPV testing, we evaluated the legitimacy of the ASC-H diagnosis. In this study, we evaluated if the ASC-H cells are related to SIL, non-SIL showing HPV infection or degenerated endocervical glandular cells.

In cases 1 to 3, which presented with SIL after the original ASC-H diagnosis, each contained small compressed binucleated squamous cells, in which HPV was detected by *in situ* PCR. In contrast, the other two cases, which remained NILM after the original ASC-H diagnosis, did not present with the small compressed binucleated squamous cells. In past studies of ASC-H, the existence of the small compressed binucleated squamous cells were not discussed as significant. The small compressed binucleated squamous cells were appearing in the background of the photos of atypical metaplastic cells in a report of Alli and Ali (2003), and those cases progressed to HSIL in follow up. Moreover, in the photos of the report that Sherman (2001) published, there were small compressed binucleated squamous cells in the background of ASC-H photos, and the follow up showed CIN2. Even though the significance of these cells are not clearly explained, we have recognized and published (Okayama et al., 2010b) that these compressed binucleated squamous cells in superficial to intermediate level of the cervical tissue tend to be associated with high risk HPV infections (*in situ* PCR). We believe that the small atypical compressed binucleated cells seen in ASC-H cases are strongly associated with presence of high-risk HPV.

The 2001 Bethesda system recommends LBC for cytological prepping. However, since LBC makes cells round up and appear smaller in liquid, it made it more difficult to detect, differentiate and diagnose small atypical cells from degenerated glandular cells, reserve cells or immature repair cells. In addition, endocervical glandular cells show binucleation as a benign change, the existence of non-compressed binucleated cells were never considered as important findings as other criteria. As we found in this study, the appearance of the compressed binucleated cells could suggest the existence of SIL with high risk HPV infection, not degenerated endocervical glandular cells. Thus, this finding of compressed binucleation can contribute to distinguish between degenerated endocervical glandular cells and ASC-H cells. In the case 4 and 5, even though they remained NILM after the original ASC-H diagnosis, it showed high risk HPV infection around the time of ASC-H diagnosis and it was considered that the small atypical cells were non-SIL showing HPV infected cells.

Since the patients of this study were not subjected to colposcopy nor histological diagnosis after ASC-H diagnosis, the determination of whether these compressed binucleated cells are related to CIN cannot be made. However, the association of small compressed binucleated squamous cells with ASC-H cannot deny the possible existence of SIL and high risk HPV infections.

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