

Thin variant of high-grade squamous intraepithelial lesion – relationship with high-risk and possibly carcinogenic human papilloma virus subtypes and somatic cancer gene mutations

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Thin variant of high-grade squamous intraepithelial lesion—relationship with high-risk and possibly carcinogenic human papilloma virus subtypes and somatic cancer gene mutations

Aim: To further characterise the thin variant of high-grade squamous intraepithelial lesions (HSILs) of the cervix defined by the World Health Organization as full-thickness HSILs with nine or fewer cell layers.

Methods and results: We examined 31 excisional cervical specimens featuring exclusively p16^{INK4a}-overexpressing thin HSILs with respect to size, location at the squamocolumnar junction or endocervical mucosa, human papilloma virus (HPV) subtypes (pretherapeutic clinical HPV tests and HPV genotyping on lesional tissue after excision), and somatic mutations in 50 cancer genes. Thin HSILs were typically solitary lesions, located at the squamocolumnar junction (20/31; 65%), in the endocervical columnar epithelium (6/31; 19%), and in both locations (5/31; 16%). The horizontal extension of thin HSILs ranged from 100 μ m to 8 mm, with 30% being <1 mm. HPV data were available for 27 specimens. Twenty of

27 (74%) thin HSILs showed high-risk HPV subtypes: HPV16 ($n = 8$), HPV16 with coinfection ($n = 2$), HPV18 ($n = 1$), HPV31 ($n = 1$), HPV33 ($n = 2$), HPV52/58 ($n = 2$), and ‘other’ high-risk HPV genotypes ($n = 4$). Five of 27 (19%) thin HSILs showed possibly carcinogenic subtypes: HPV53 ($n = 3$), HPV73 ($n = 1$), and HPV82 ($n = 1$). One thin HSIL was induced by low-risk HPV6 and one by the unclassified subtype HPV44. Somatic gene mutations were not identified.

Conclusion: Thin HSILs were typically small lesions without somatic gene mutations. Two-thirds of thin HSILs developed after a transforming infection with high-risk HPV subtypes, and one-third were induced by non-high-risk HPV subtypes. If cervical cancer screening relies solely on presently available clinical HPV DNA tests, a significant percentage of women with HSIL will be missed.

Keywords: cancer hotspot panel, cervical carcinogenesis, cervical intraepithelial neoplasia, HPV cervical cancer screening, p16^{INK4a} overexpression, somatic gene mutations

Introduction

Invasive cervical squamous cell carcinoma arises from the precancerous lesion high-grade squamous

intraepithelial lesion (HSIL) after a transforming infection with high-risk human papilloma virus (HPV) genotypes.^{1,2} This has led to the development of clinical (screening) HPV tests, comprising 12 of the most common high-risk HPV subtypes.³ Several variants of HSIL are recognised by the World Health Organization, the most common being the classic ‘thick’ HSIL with a thickness of ≥ 10 cell layers (synonymous with cervical intraepithelial neoplasia III).

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Rare variants are thin HSIL that is nine or fewer cells thick, condylomatous HSIL, keratinising HSIL, and papillary HSIL.^{4–6} Thick and thin HSILs often coexist in surgical specimens, but the occurrence of isolated thin HSILs is rarely observed in excisional specimens of the cervix uteri. Despite the recognition of thin HSILs in various classifications,^{4,5} detailed studies of thin HSILs regarding location, size, HPV genotyping and cancer gene analysis are lacking. The results of a quality control analysis involving a cone specimen with a thin p16^{INK4a}-overexpressing HSIL and a preceding negative HPV DNA test result revealing the possibly carcinogenic HPV53 subtype as the single HPV subtype prompted us to search for other excisional specimens with thin HSILs only. We identified 31 patients with exclusively thin HSILs, which were genotyped, and analysed for size and location, and the presence of somatic mutations in 50 genes (cancer hotspot panel).

Materials and methods

From August 2015 to August 2018, we identified 31 excisional specimens with a histological diagnosis of thin HSIL only as compared with 500 thick or mixed HSILs, corresponding to a 6% prevalence at the time of treatment. The complete excisional specimens were submitted for histological examination, resulting in 6–10 blocks with 5–10 serial sections cut from each block. For each lesion, at least one p16 immunohistochemical stain was performed. In some cone specimens, up to four blocks were stained, to exclude immature metaplasia. This type of work-up was responsible for the fact that—despite larger lesions of thin HSIL—some blocks yielded insufficient tissue for analysis.

p16^{INK4a} overexpression (Roche-mtm Laboratories, Heidelberg, Germany) in the thin HSIL was required as an indirect marker for a transforming infection, and was defined as homogeneous continuous strong nuclear and cytoplasmic staining of the entire dysplastic epithelium, beginning in the basal and parabasal cells, and extending continuously to the superficial layers of the dysplastic epithelium (also referred to as ‘block’ staining). Discontinuous and patchy staining—independent of the percentage of positivity—was interpreted as negative.⁷

Human papilloma virus DNA data were obtained either from HPV genotyping or from HPV DNA tests prior to conisation. A liquid-based cervical cytology sample (ThinPrep; Hologic, Bedford, MA, USA) was obtained for HPV testing with either the Roche Cobas

HPV Test (Roche Molecular Systems, Pleasanton, CA, USA) or the Digene HC2 High-Risk HPV DNA Test (Qiagen, Hilden, Germany). The Roche/Cobas HPV DNA test reports individual results for HPV16 and HPV18 and, simultaneously, 12 pooled high-risk genotypes as ‘other’ (HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68). The Digene HC2 High-Risk HPV DNA Test lists individual HPV subtypes.

Human papilloma virus genotyping was performed on microdissected tissue of formalin-fixed paraffin-embedded lesional tissue. The assay is based on two polymerase chain reactions (PCRs) that are combined prior to hybridisation into one array field. Both primer mixes are directed against highly conserved motifs within the viral L1 gene. Primer mix A generates fragments of ~450 bp in length (HPV type-dependent), and primer mix B generates amplicon sizes of ~165 bp (HPV type-dependent). SPF10 is a PCR-based technique that detects a 65-bp region in the L1 open reading frame of the DNA of the virus. Combining both independent single PCRs prior to hybridisation into one array field ensures the parallel and robust detection of all 32 HPV types at five target copies per reaction for HPV16, HPV18, and HPV31, and at 50 target copies for all other HPV types (Chiron, Berlin, Germany): high-risk HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59; low-risk HPV subtypes 6 and 11; probably carcinogenic HPV subtype 68; possibly carcinogenic HPV subtypes 26, 53, 66, 67, 70, 73, and 82; and the unclassified HPV subtypes 42, 44, 54, 61, 62, 72, 81, 84, 90, and 91. DNA was extracted on a Maxwell MDx Research System (Promega, Fitchburg, WI, USA).

Thin HSILs with a single possibly carcinogenic HPV subtype were subjected to next-generation sequencing (NGS) analysis. NGS libraries were prepared with the AmpliSeq library kit 2.0 (Life Tech Austria, Vienna, Austria) and the Ion Ampliseq Cancer Hotspot Panel V2 (Cat. No: 4475346) primer pool, covering hotspot mutations in 50 genes implicated in cancer. Sequencing was performed on an Ion Proton benchtop sequencer (Thermo Fisher Scientific) to a length of 200 bp. Initial data analysis was performed with the ION TORRENT suite software plug-ins (Thermo Fisher Scientific; open source, GPL, <https://github.com/iontorrent/>). Briefly, this included base calling, alignment to the reference genome (HG19) by use of the TMAP mapper, and variant calling with a modified diBayes approach, taking into account the flow space information. Called variants were annotated by the use of the open source software ANNOVAR⁸ and SNPEFF.⁹ All coding, non-synonymous mutations

were further evaluated and visually inspected in IGV (<http://www.broadinstitute.org/igv/>), and variant calls resulting from technical read errors or sequence effects were excluded from the analysis.

Institutional review board approval was obtained (31-049 ex. 18/19).

Results

HISTOLOGICAL FEATURES

We identified 31 patients (age range, 19–58 years; median, 33 years) with isolated p16^{INK4a}-overexpressing thin HSILs in the absence of other HSIL variants (Table 1). Twenty-seven of 31 (87%) thin HSILs were solitary lesions and were located on the surface, and not in crypts. Five of 31 (16%) specimens showed multiple lesions of thin HSIL at the squamocolumnar junction and in the endocervical epithelium. Six of 31 (19%) thin HSILs were identified only within the endocervical epithelium; that is, they were bordered by a row of non-neoplastic mucin-producing columnar epithelium. When measured on haematoxylin and eosin (H&E)-stained or immunohistochemically stained sections, the horizontal extension ranged from 100 µm to a maximum of 8 mm (Figures 1A–H and 2A–F), with seven of 31 (29%) thin HSILs being <1 mm (Figures 1A–F and 2A,B,E,F). Eighteen of 31 (58%) thin HSILs were between 1 and 3 mm, and six of 31 (19%) thin HSILs were ≥4 mm (Figure 1G,H). Twenty of 31 (65%) thin HSILs were located at the squamocolumnar junction, six of 31 (19%) in the endocervical columnar epithelium, and five of 31 (16%) in both locations. HSILs detected in the endocervical epithelium were bordered by a row of non-neoplastic mucin-producing columnar epithelium.

HPV GENOTYPING AND MUTATIONAL ANALYSIS OF CANCER GENES

Human papilloma virus data were obtained by genotyping of microdissected, formalin-fixed paraffin-embedded tissues of thin HSIL (16 of 31 patients) and/or by pretherapeutic clinical HPV DNA tests (21 of 31 patients). In two of 31 (6%) thin HSILs without preceding HPV tests and another two of 31 (6%) thin HSILs with negative clinical HPV test results, the HPV subtype could not be determined, because of an insufficient amount of DNA (no lesional tissue left in the block after serial sectioning of blocks) or poor DNA quality (patients 28–31), leaving 27 patients for further analysis. In 20 of the remaining 27 thin HSILs (74%), high-risk HPV subtypes were

demonstrated. HPV16 was present in 10 of 27 (37%) thin HSILs, either as the single subtype HPV16 ($n = 8$; Figure 2C–E) or as coinfection ($n = 2$). HPV18 in one of 27 thin HSILs accounted for only 4% (Figure 2A,B). High-risk HPV subtypes other than HPV16/18 were isolated from five of 31 thin HSILs (16%; Figure 1C,D): HPV31 ($n = 1$), HPV33 ($n = 2$), HPV51/52/58 ($n = 1$), and HPV52/58 ($n = 1$). Possibly carcinogenic HPV subtypes were identified in five of 27 (19%) thin HSILs: HPV53 ($n = 3$; Figure 2F), HPV73 ($n = 1$; Figure 2G), and HPV82 ($n = 1$; Figure 1E,F). One of 27 thin HSILs each was induced by low-risk HPV6 (Figure 1H) and the not further classified subtype HPV44. In summary, a total of nine of 27 (33%) thin HSILs were not detected by clinical DNA tests (seven thin HSILs with genotyping results, and two thin HSILs without genotyping results because of insufficient DNA). None of the thin HSILs showed somatic gene mutations.

CORRELATION OF LOCATION AND HISTOLOGY OF THIN HSILS WITH HPV SUBTYPES

There were no histological differences or differentiating criteria allowing distinction of thin HSILs induced by high-risk HPV and those induced by non-high-risk HPV subtypes on H&E-stained sections. Furthermore, no specific HPV subtype could be associated with the location of thin HSILs. High-risk HPV-induced thin HSILs were identified in both the squamocolumnar junction and the endocervical columnar epithelium. The six thin HSILs that were identified exclusively in the endocervical columnar epithelium showed predominantly high-risk HPV [HPV16 ($n = 2$), and one each of HPV33, HPV51/52/58, and HPV53].

Discussion

The findings on thin HSILs presented in this article have consequences for cervical cancer screening and diagnostic procedures. Only three-quarters of thin HSILs were induced by high-risk HPV subtypes. The high percentage of 25% of non-high-risk HPV (possibly carcinogenic HPV subtypes were much more common than low-risk HPV subtypes and unclassified HPV subtypes) explains the negative pretherapeutic HPV DNA test results. Similarly to the results of a meta-analysis in >100 000 women with cervical precancerous lesions,¹⁰ HPV16 was also the most common subtype detected in thin HSILs (37%), followed by the phylogenetically related α9 high-risk non-HPV16/18 (epidermotropic) subtypes (33%). With only one exception of an HPV18-induced thin HSIL,

Table 1. Thin high-grade squamous intraepithelial lesion (HSIL) clinical and molecular data

Patient	Age at diagnosis (years)	Clinical HPV DNA testing	HPV genotype (SPF10)	NGS cancer hotspot panel	Location of thin HSIL	Horizontal diameter of thin HSIL (mm)	
1	25	16	ND	ND	SCJ	0.5	
2	32	16	ND	ND	Endocervical epithelium	1.5	
3	24	16	ND	ND	Endocervical epithelium	0.1–0.3	Figure 2C–E
4	19	16	ND	ND	SCJ	0.5	
5	58	16	16	Negative	SCJ	8	
6	32	ND	16	Negative	SCJ	2.5	
7	47	ND	16	Negative	SCJ	7	
8	34	ND	16	Negative	SCJ and endocervical epithelium	7	
9	34	16 and 'other'	Insufficient tissue	Insufficient tissue	SCJ and endocervical epithelium	0.3 and 0.35	Figure 1A, B
10	29	16 and 'other'	ND	ND	SCJ	3	
11	25	18	ND	ND	SCJ and endocervical epithelium	1.1–2.2	Figure 2A, B
12	22	51, 52, 58	ND	ND	Endocervical epithelium	2	
13	19	ND	52, 58	Negative	SCJ	4	
14	35	'Other'	31	Negative	SCJ	3	
15	40	'Other'	33	Negative	Endocervical epithelium	1.5	
16	41	ND	33	Negative	SCJ	2	
17	24	'Other'	Insufficient tissue	Insufficient tissue	SCJ	3	Figure 1C, D
18	30	'Other'	Insufficient tissue	Insufficient tissue	SCJ	1	
19	23	'Other'	Insufficient tissue	Insufficient tissue	SCJ	1.2	
20	46	'Other'	Insufficient tissue	Insufficient tissue	Endocervical epithelium	1	
21	23	Negative	82	Negative	SCJ	1–2.5	Figure 1E, F
22	53	Negative	73	Negative	SCJ and endocervical epithelium	0.5	Figure 1G
23	36	ND	53	Negative	SCJ and endocervical epithelium	2	Figure 2F
24	28	Negative	53	Negative	SCJ	1.5	
25	52	ND	53	Negative	Endocervical epithelium	6	
26	19	Negative	6	Negative	SCJ	1	Figure 1H

Table 1. (Continued)

Patient	Age at diagnosis (years)	Clinical HPV DNA testing	HPV genotype (SPF10)	NGS cancer hotspot panel	Location of thin HSIL	Horizontal diameter of thin HSIL (mm)
27	52	ND	44	Negative	SCJ	4
28	41	Negative	Insufficient tissue		SCJ	0.8
29	45	Negative	Insufficient tissue		SCJ	0.5
30	30	ND	Insufficient tissue		SCJ	6
31	37	ND	Insufficient tissue		SCJ	1.5

HPV, Human papilloma virus; ND, Not done; NGS, Next-generation sequencing; SCJ, Squamocolumnar junction.

no $\alpha 7$ high-risk (mucosal) HPV subtypes were detected.

Most models of HPV-induced carcinogenesis are based on HPV16 subtype-induced transformation of mature stratified squamous epithelium, resulting in thick HSILs.¹¹ It is unclear at present how thin HSILs develop; that is, whether they arise directly *de novo* and develop into thick HSILs over time, or whether they represent regressed thick HSILs. Thin HSILs at the squamocolumnar junction arise from infected squamocolumnar junction cells or immature metaplastic epithelium. Thin HSILs in the endocervical epithelium most likely arise from reserve cells, thus contributing to difficulties in recognising the neoplastic nature of thin HSILs on H&E-stained sections. Thin HSIL may be mistaken for immature metaplasia with mild atypia or atypical immature metaplasia, but immunohistochemical p16^{INK4a} overexpression can assist in the unequivocal diagnosis of thin HSIL in this setting.^{12,13} The differentiation and maturation level of the infected epithelium at the time of transformation determines which variant of HSIL develops and is detected at the time of excision.

The minute size of thin HSILs in excisional specimens of the cervix uteri may explain the difficulties in colposcopic detection, and the failure to detect them in clinical HPV tests as a result of low copy numbers of HPV/insufficient amounts of HPV DNA or sampling artefacts. On the other hand, the majority of these negative cases were related to group 2B/possibly carcinogenic HPV subtypes, which are not included in the clinical HPV tests. These lesions, however, were detected by cytology or biopsy subsequent to suspicious abnormal colposcopic findings. In our Austrian/European patient population with isolated thin HSILs, HPV53 was the most common subtype, matching observations on HSILs in general in Italy.¹⁴ In a US

study, however, HPV82 was the most common group 2B HPV subtype in precancerous lesions.¹⁵ Possibly carcinogenic HPV subtypes affect the same cellular pathways as any of the fully recognised carcinogenic high-risk HPV subtypes,¹⁶ and both HPV53 and HPV82 are able to immortalise human keratinocytes.¹⁷ On the basis of these results, inclusion of these two subtypes in HPV tests should be discussed.

Many invasive cervical cancers also harbour somatic gene mutations, such as mutations in *PIK3CA*, *TP53*, *STK11*, and *MAPK1*. They are considered to be the necessary additional driver hits for progression of HSIL to invasive cancer.^{18–21} In the absence of somatic gene mutations, possibly carcinogenic HPV subtypes have to be considered to be the sole cause of the development of thin HSILs. The natural history of a thin HSIL arising *de novo*, however, is unknown, particularly the risk of progression to invasion of thin HSILs induced by possibly carcinogenic HPV subtypes. The discrepancy between a 19% prevalence of possibly carcinogenic HPV subtypes in thin HSILs in our study and only a 3% cumulative worldwide prevalence of possibly carcinogenic HPV subtypes in invasive cervical cancer¹⁶ suggests that HSILs induced by possibly carcinogenic HPV subtypes have a low risk of progression to invasive cancer.

In conclusion, thin HSILs are small to minute lesions that develop after transforming infections with epidermotropic high-risk HPV, but also after infections with non-high-risk, mostly possibly carcinogenic HPV subtypes in the absence of somatic gene mutation. The natural biology of thin HSILs associated with possibly carcinogenic HPV subtypes remains unclear, but it is likely that they show less aggressive behaviour than classic HSIL. Presently available clinical HPV DNA tests, which detect high-risk HPV subtypes only, will miss a certain percentage of women

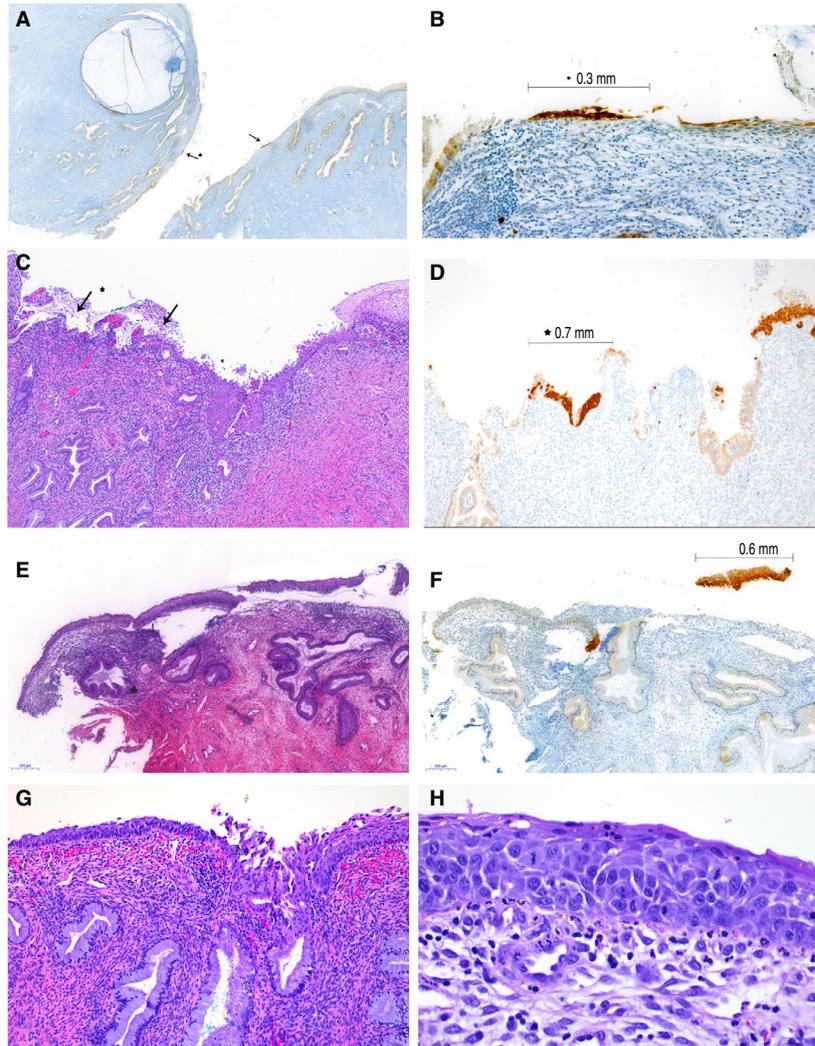


Figure 1. A thin high-grade squamous intraepithelial lesion (HSIL) arising at the squamocolumnar junction. A,B, Patient 9; HPV16 with coinfection. A, Immunohistochemical stain with an antibody against p16^{INK4a}. This low-power view of a cone specimen shows two minute foci of a thin HSIL (arrows). B, High-power view of the area indicated with an asterisk in (A), revealing a thin HSIL with a thickness of two to four cell layers. C,D, Patient 17; clinical HPV test 'other'; insufficient tissue for genotyping. C, Haematoxylin and eosin stain of a cone specimen featuring transition of mature squamous epithelium to endocervical columnar epithelium. The area between arrows features the squamocolumnar junction and endocervical mucosa. The area denoted with an asterisk is highlighted in (D); an immunohistochemical stain with antibody against p16^{INK4a} demonstrates a thin HSIL with two to four cell layers. E,F, Patient 21; HPV82. E, A thin HSIL at the squamocolumnar junction arising next to immature squamous epithelium. F, A p16^{INK4a} immunohistochemical stain identifies a detached dysplastic epithelium. G, Patient 22; HPV73. A thin HSIL with two to three cell layers arising adjacent to metaplastic squamous epithelium of the squamocolumnar junction, H, Patient 26; HPV6. A thin HSIL with four to six cell layers with mitotic activity. HPV, human papilloma virus.

with HSIL if cancer screening relies solely on presently available HPV DNA screening tests.

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Conflicts of interest

The authors state that they have no conflicts of interest.

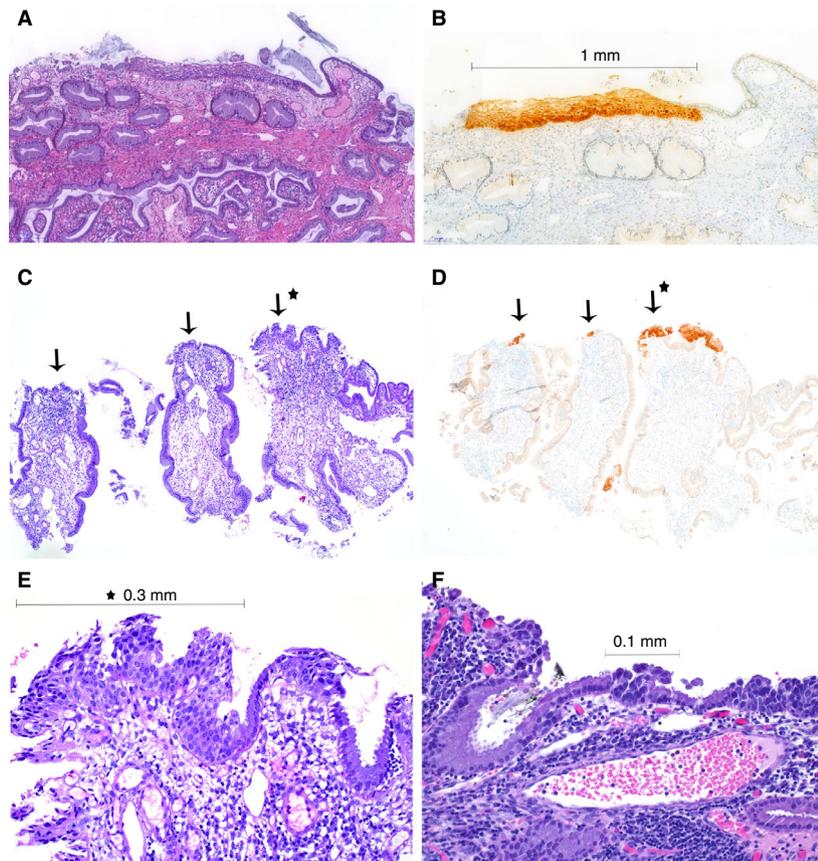


Figure 2. A thin high-grade squamous intraepithelial lesion (HSIL) arising in the endocervical epithelium. A,B, Patient 11; HPV18. A, A 1-mm large focus of a thin HSIL resembling squamous metaplasia is bordered by a single row of endocervical epithelium. B, p16^{INK4a} overexpression identifies this immature lesion as a thin HSIL. C–E, Patient 3; HPV16. C, Three minutes foci of a thin HSIL (arrows) are bordered by endocervical columnar epithelium (D), confirmed by immunohistochemical overexpression of p16^{INK4a}, which is an indirect marker of a transforming HPV infection (arrows). E, High-power view of a haematoxylin and eosin-stained histological section. The thin HSIL resembles immature metaplasia. Cytological atypia is mild, with slight anisokaryosis in occasional nuclei. Mitotic activity is absent. F, Patient 23; HPV53. High-power view of a minute focus of a thin HSIL arising in the endocervical mucosa. HPV, human papilloma virus.

Author contributions

S. Regauer designed the study, analysed data, compiled figures, and wrote the manuscript. K. Kashofer analysed and interpreted the results of HPV genotyping and cancer hotspot analysis. O. Reich performed the clinical examinations, including clinical HPV tests, and assisted with writing of the manuscript. All authors have seen the final version of the manuscript.

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