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RESEARCH

p16^{INK4a} Immunoprofiles of Squamous Lesions of the Uterine Cervix–Implications for the Reclassification of Atypical Immature Squamous Metaplasia

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Abstract p16^{INK4a} immunoprofiles of non-precancerous and dysplastic squamous cervical lesions were defined and applied to the reclassification of atypical immature squamous metaplasia (AIM). The immunoexpression of cytokeratin 17 (CK 17) in AIM was also evaluated. Totally, 295 cervical cone biopsies representing squamous metaplasia, reactive changes, koilocytosis, flat condyloma, CIN I, CIN II, CIN III and AIM were subjected to p16^{INK4a} immunohistochemistry. AIM cases were analyzed using CK 17 antibody. Typical p16^{INK4a} immunoprofiles for the metaplastic, LSIL/HPV and HSIL phenotypes were recorded and used for the categorization of AIM into particular phenotype groups. Results were correlated with CK 17 immunoexpression. All CIN II and CIN III lesions, all but one case of CIN I and all flat condylomas overexpressed p16^{INK4a}. Other non-precancerous lesions, including koilocytosis, were predominantly negative. Contrary to the sporadic and focal immunostaining, diffuse positivity was associated with the dysplastic features of the lesion. CIN II and CIN III were characterized by a diffuse, strong/weak, full-thickness staining, whereas CIN I showed a heterogeneous diffuse/focal, weak/strong, lower half positivity. One third of AIM lesions may be reclassified as HSIL, one third as LSIL/HPV and one third shows metaplastic phenotype. All AIM cases with metaplastic and LSIL/HPV phenotypes expressed CK 17 diffusely, whereas focal positivity slightly prevailed in AIM with HSIL phenotype. We conclude that p16^{INK4a} immunohistochemistry is a supporting method for the differential diagnosis of cervical lesions, which may be especially useful for the reclassification of AIM. The efficacy of CK 17 immunohistochemistry seems to be controversial for these purposes.

Keywords Uterine cervix · p16 · Cytokeratin 17 · Cervical intraepithelial neoplasia · Atypical immature squamous metaplasia · Flat condyloma

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Introduction

Cervical intraepithelial neoplasias (CIN) are traditionally classified into three grades: CIN I–III, or an alternative terminology of low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) may be applied. Unfortunately, the histopathological evaluation of cervical biopsies may be influenced by a significant inter- and intraobserver variation [1] that affects especially CIN I [2] and CIN II [3] categories. Further differential diagnostic issues emerge because of a spectrum of benign lesions, which may mimic cervical dysplasias microscopically. One of the most enigmatic entities from this group, initially described by Crum et al. [4], is the atypical immature squamous metaplasia (AIM). It probably represents a heterogeneous group of lesions of various precancerous potential, including LSIL, HSIL and reactive or inflammatory conditions [5–7]. Regrettably, its biologic behavior and clinical significance as a diagnostic category remain unclear.

Cyclin-dependent kinase inhibitor p16^{INK4a}, which is involved in the regulation of cell cycle, may be overexpressed as a consequence of infection with oncogenic high-risk human

papillomavirus (HR-HPV). The immunostaining for $p16^{INK4a}$ is therefore a feature of HPV-associated precancerous lesions and carcinomas of the lower female genital tract [8] and it may be used as an auxiliary method for histopathological evaluation. It was also shown that the level of $p16^{INK4a}$ upregulation correlates with the increasing grade of CIN [9].

Cytokeratin 17 (CK 17) was identified as a marker of cervical stem cells [10], it is consistently expressed in immature squamous metaplasia of the cervical transformation zone [11] and its immunoexpression was also observed in all grades of CIN [11, 12]. However, the prevalent negativity of CK 17 in CIN III was reported and the suggestion to use the reciprocal immunoreactivity of $p16^{INK4a}$ and CK 17 for the distinction between AIM and high-grade CIN was postulated [13].

In this study, we primarily aimed to analyze the expression of $p16^{INK4a}$ in a broad spectrum of squamous lesions of the uterine cervix with various precancerous potential: non-precancerous proliferations (squamous metaplasia, reactive changes), LSIL/HPV group (koilocytosis, flat condylomas, CIN I), HSIL group (CIN II, CIN III) and AIM. Our objective was to estimate typical $p16^{INK4a}$ immunoprofiles of each type of lesion and to define their diagnostic value for the potential reclassification of AIM. Secondarily, the immunoexpression of CK 17 in all cases of AIM was performed to assess its role in the differential diagnosis between AIM and high-grade CIN.

Materials and Methods

Case Selection

In total, 351 cervical cone biopsies were included into the study. Incomplete cone excisions and punch biopsies were discarded because they often fail to demonstrate a representative portion of the transformation zone for the evaluation. Slides were reviewed independently by two consultant pathologists (P.S. and J.Z.): only cases with the concurrent diagnostic interpretation from both observers were enrolled into the study. Finally, 295 cone biopsies were available for the analysis and 56 cases were excluded due to the diagnostic disagreement. All lesions were classified into the following groups: mature and immature squamous metaplasia (SM, $n=33$), metaplastic squamous epithelium with reactive changes (RC, $n=23$), metaplastic squamous epithelium with koilocytosis (Kc, $n=15$), flat condyloma (Co, $n=8$), CIN I ($n=35$), CIN II ($n=82$), CIN III ($n=67$) and AIM ($n=32$). Generally accepted histopathological criteria were used for the classification of SM, RC, Co, CIN I, CIN II and CIN III. Koilocytosis was defined as a non-dysplastic, non-acanthotic and non-papillomatous squamous epithelium containing monucleated or multinucleated cells with perinuclear halos, nuclear enlargement and irregular nuclear contours. The criteria proposed by Crum et al. [4] were applied to the diagnosis of AIM. Provided that more lesions with a different biologic behavior were present in one specimen, only the lesion with the highest precancerous potential was considered for further analysis.

Immunostaining Protocols

Tissue sections intended for $p16^{INK4a}$ immunohistochemistry were subjected to the heat-induced epitope retrieval in water bath at 98 °C for 30 min and incubated overnight at 4 °C with primary monoclonal mouse anti-human antibody $p16^{INK4a}$ (diluted 1:100) (clone G175-405, cat. No. 551154, BD Biosciences, Franklin Lakes, NJ). The immunocomplexes of the antigen and the primary antibody were visualized using N-Histofine Simple Stain MAX PO (MULTI) detection system (cat. No. 414154F, Nichirei Biosciences, Tokyo, Japan). The positive control (squamous cell carcinoma of the uterine cervix) was used in each series of immunohistochemistry.

Nuclear staining or a combination of nuclear and cytoplasmic staining was considered for a positive result of immunoreaction with $p16^{INK4a}$ antibody. Cytoplasmic staining without nuclear staining was interpreted as negativity. All cases were reviewed by two observers and consensually assessed according to the scoring system summarized in Table 1. Three basic parameters were used for evaluation of the immunoreaction: horizontal distribution, vertical distribution and intensity. The horizontal distribution of staining was

Table 1 A standardized scoring system used in this study for the evaluation of $p16^{INK4a}$ immunostaining

Parameter	Value	Histopathological criteria
Horizontal distribution	Negative	Positivity of solitary cells (<1 %)
	Sporadic	Positivity of solitary cells (≥1 % and <5 %)
	Focal	Positivity of solitary cells or clusters of cells (≥5 % and <25 %)
	Diffuse	Band-like confluent positivity (≥25 %)
Vertical distribution	Lower half	Horizontal staining pattern contained to the lower half of the epithelium
	Full-thickness	Horizontal staining pattern extending above the lower half of the epithelium
Intensity	Weak	Light brown staining of substantially lower intensity than a positive control sample, nuclear membranes clearly visible, chromatin pattern distinguishable
	Strong	Dark brown staining comparable with a positive control sample, nuclear membranes and chromatin pattern poorly recognizable

scored according to Klaes et al. [14]. The vertical distribution was interpreted on the basis of maximal vertical alignment of the horizontal staining pattern. The two-grade scoring system (lower half and full-thickness positivity) was used for this purpose instead of the three-grade scheme (lower third, middle third and full-thickness positivity) to ensure the sufficient standardization and reproducibility of the histologic assessment. The comparison with a positive control sample was applied for the evaluation of staining intensity. The three basic parameters of staining were combined into 13 possible immunoprofiles (summarized in Fig. 1).

Tissue sections intended for CK 17 immunohistochemistry were immersed in Target Retrieval Solution (cat. No. S 1700, DakoCytomation, Glostrup, Denmark) for the epitope retrieval at 98 °C for 30 min and subsequently incubated overnight at 4 °C with primary monoclonal mouse anti-human antibody Cytokeratin 17 (diluted 1:100) (clone E3, cat. No. M 7046, DakoCytomation, Glostrup, Denmark). The immunocomplexes of the antigen and the primary antibody were visualized using the streptavidin-biotin detection kit LSAB+, Dako REAL™ Detection Systems, HRP/DAB+, Rabbit/Mouse (cat. No. K 5001, DakoCytomation, Glostrup, Denmark). The positive control (immature squamous metaplasia of the uterine cervix) was used in each series of immunohistochemistry.

All slides immunostained for CK 17 were reviewed by two observers and consensually evaluated. Cytoplasmic staining was considered for a positive result of the immunoreaction. The two-grade scoring system was used to scale the extent of CK 17 staining. Focal positivity was defined as a non confluent staining of single cells or clusters of cells and the diffuse positivity corresponded with a confluent band-like staining.

Results

p16^{INK4a} Immunoprofiles of Lesions

The rates of p16^{INK4a} positive cases in particular groups of patients are calculated in Table 2 and the frequency of all 13 possible immunoprofiles is shown in Fig. 1. Samples of p16^{INK4a} immunostaining are exemplified in Figs. 2 and 3. p16^{INK4a} negativity prevailed in the SM (87.9 %), RC (78.4 %) and Kc (60.0 %) groups. All cases from the Co group were p16^{INK4a} positive and the typical immunostaining profile was a focal, weak, full-thickness positivity (87.5 %). Although the majority of lesions from the CIN I group were p16^{INK4a} positive (97.1 %), their immunoprofiles were diverse and showed mostly diffuse positivity of varying intensity in the lower half of the epithelium (71.4 %) and a focal, weak, lower half staining (17.1 %). The most common p16^{INK4a} immunoprofile in the CIN II and CIN III groups was a diffuse, strong, full-thickness positivity (84.1 % in the CIN II and 94.0 % in the CIN III group). p16^{INK4a} positive lesions from the AIM group (68.8 %) showed four immunoprofiles of approximately similar frequencies (none of these immunoprofiles significantly prevailed).

CK 17 Immunoexpression in AIM

In total, 31 cases of AIM were available for CK 17 immunohistochemistry. One lesion with a diffuse, strong, full-thickness p16^{INK4a} positivity was lost during the previous serial sectioning. The immunoexpression of CK 17 was observed in all AIM lesions. The majority (80.6 %) showed diffuse staining which was usually intense and affected full-thickness of the epithelium. Focal positivity (19.4 %) of single cells or clusters of cells was typically limited to the basal zones of the epithelium and its intensity was more heterogeneous. The diffuse CK 17 staining was constantly observed in all AIM lesions which were p16^{INK4a} negative or showed low level of p16^{INK4a} expression (sporadic, weak, full-thickness and focal, weak, full-thickness staining). In the group of AIM lesions with diffuse, strong, full-thickness p16^{INK4a} positivity, 50.0 % of cases showed diffuse CK 17 immunoexpression and 50.0 % of lesions were focally positive. All AIM cases with focal, strong, full-thickness p16^{INK4a} positivity were focally stained for CK 17. An overview of CK 17 immunostaining in particular groups of lesions is calculated in Table 3 and typical samples are exemplified in Fig. 3.

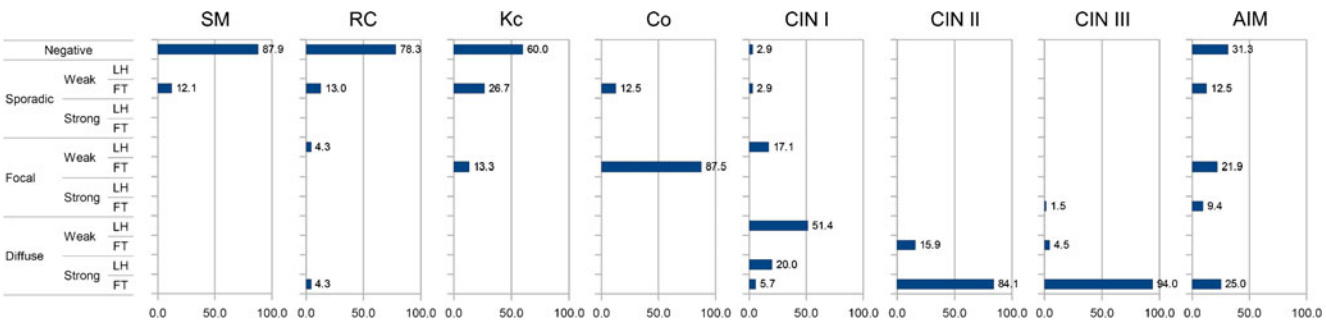

Fig. 1 Percentage frequencies of 13 possible p16^{INK4a} immunoprofiles in particular groups of lesions. *LH* lower half, *FT* full-thickness

Table 2 An overview of $p16^{INK4a}$ positivity and the spectrum of $p16^{INK4a}$ immunoexpression patterns in particular groups of lesions

Lesion	Number of $p16^{INK4a}$ positive cases	$p16^{INK4a}$ positivity (%)	$p16^{INK4a}$ staining parameters in positive cases (%)						
			Horizontal distribution			Vertical distribution		Intensity	
			Sporadic	Focal	Diffuse	Weak	Strong	Lower half	Full-thickness
SM	4	12.1	100.0	–	–	100.0	–	–	100.0
RC	5	21.6	60.0	20.0	20.0	80.0	20.0	20.0	80.0
Kc	6	40.0	66.7	33.3	–	100.0	–	–	100.0
Co	8	100.0	12.5	87.5	–	100.0	–	–	100.0
CIN I	34	97.1	2.9	17.7	79.4	73.5	26.5	91.2	8.8
CIN II	82	100.0	–	–	100.0	15.9	84.1	–	100.0
CIN III	67	100.0	–	1.5	98.5	4.5	95.5	–	100.0
AIM	22	68.8	18.2	45.4	36.4	50.0	50.0	–	100.0

SM mature and immature squamous metaplasia, *RC* metaplastic squamous epithelium with reactive changes, *Kc* metaplastic squamous epithelium with koilocytosis, *Co* flat condyloma, *CIN I* cervical intraepithelial neoplasia I, *CIN II* cervical intraepithelial neoplasia II, *CIN III* cervical intraepithelial neoplasia III, *AIM* atypical immature squamous metaplasia

Discussion

$p16^{INK4a}$ Immunoprofiles of Lesions

Squamous dysplastic lesions of the uterine cervix are generally considered $p16^{INK4a}$ positive, although the results differ between studies according to the grade of lesions and the immunoscoring system used. The highest heterogeneity was seen in the CIN I category, where the rate of $p16^{INK4a}$ expression varied between 35 % [9] and 100 % [15]. Although $p16^{INK4a}$ positivity of the lesions from the CIN II and CIN III categories reached mostly 90–100 % [14, 15], a higher proportion of negative lesions (up to 33 %) has been reported [9]. All but one case of CIN I and all lesions from the CIN II and CIN III groups were $p16^{INK4a}$ positive in our series. Similar to Sano et al. [16], we observed $p16^{INK4a}$ immunoexpression in all cervical flat condylomas. A relatively high rate of $p16^{INK4a}$ negative cases of koilocytosis (60.0 %) in comparison with CIN I and flat condylomas might be explained by different HPV-mediated molecular events in these lesions or by poor reproducibility of koilocytosis.

Our results indicate that the diffuse $p16^{INK4a}$ positivity is strongly associated with the dysplastic behavior of the lesion: it was observed in 98.5 % of CIN III, in 100.0 % of CIN II and in 77.1 % of CIN I, but it was not present in the SM, Kc and Co groups and only one case from the RC group showed this immunostaining pattern. Diffuse staining in the CIN I group was mostly weak (51.4 %), sometimes strong (25.7 %), but predominantly limited to the lower half of the epithelium, whereas it was usually strong and extended to the upper parts of the epithelium in the CIN II and CIN III groups. Similar immunoprofiles of particular grades of CIN were reported in previous studies [14–17]. Focal expression of $p16^{INK4a}$ was observed in our study especially in non-dysplastic lesions associated with HPV infection (87.5 % of Co and 13.3 % of Kc). It occurred in 17.1 % of CIN I as well, but it was only rarely seen in other types of lesions (one case from the RC and CIN III groups). Similar patterns of focal $p16^{INK4a}$ positivity in condylomas and CIN I were detected previously [14, 16], although some papers describe explicitly diffuse positivity in the CIN I category without any focal staining [18]. Our data further showed that sporadic expression of $p16^{INK4a}$ is strongly associated with a non-precancerous behavior of such a lesion. It was observed in the SM (12.1 %), RC (13.0 %), Kc (26.7 %) and Co (12.5 %) groups (only one case of CIN I showed this pattern). Sporadic $p16^{INK4a}$ expression in non-precancerous lesions, including condylomas, was also well documented in previous studies [14–16].

The classification of squamous lesion of the uterine cervix into one of the three diagnostic groups: no dysplasia, LSIL and HSIL, represents the sufficient and clinically relevant information for the appropriate treatment of a patient [19]. These basic phenotypes could be defined in our study as follows: metaplastic (represented by combined SM and RC groups), LSIL/HPV (represented by combined Kc, Co and CIN I groups) and HSIL (represented by combined CIN II and CIN III groups). Our data indicate that the typical $p16^{INK4a}$ immunoprofile of the metaplastic phenotype is negativity. LSIL/HPV phenotype shows a heterogeneous pattern of $p16^{INK4a}$ immunoexpression which may be best defined as a sporadic, weak, full-thickness positivity or a focal, weak, lower half/full-thickness positivity or a diffuse, weak/strong, lower half positivity, where all lesions with

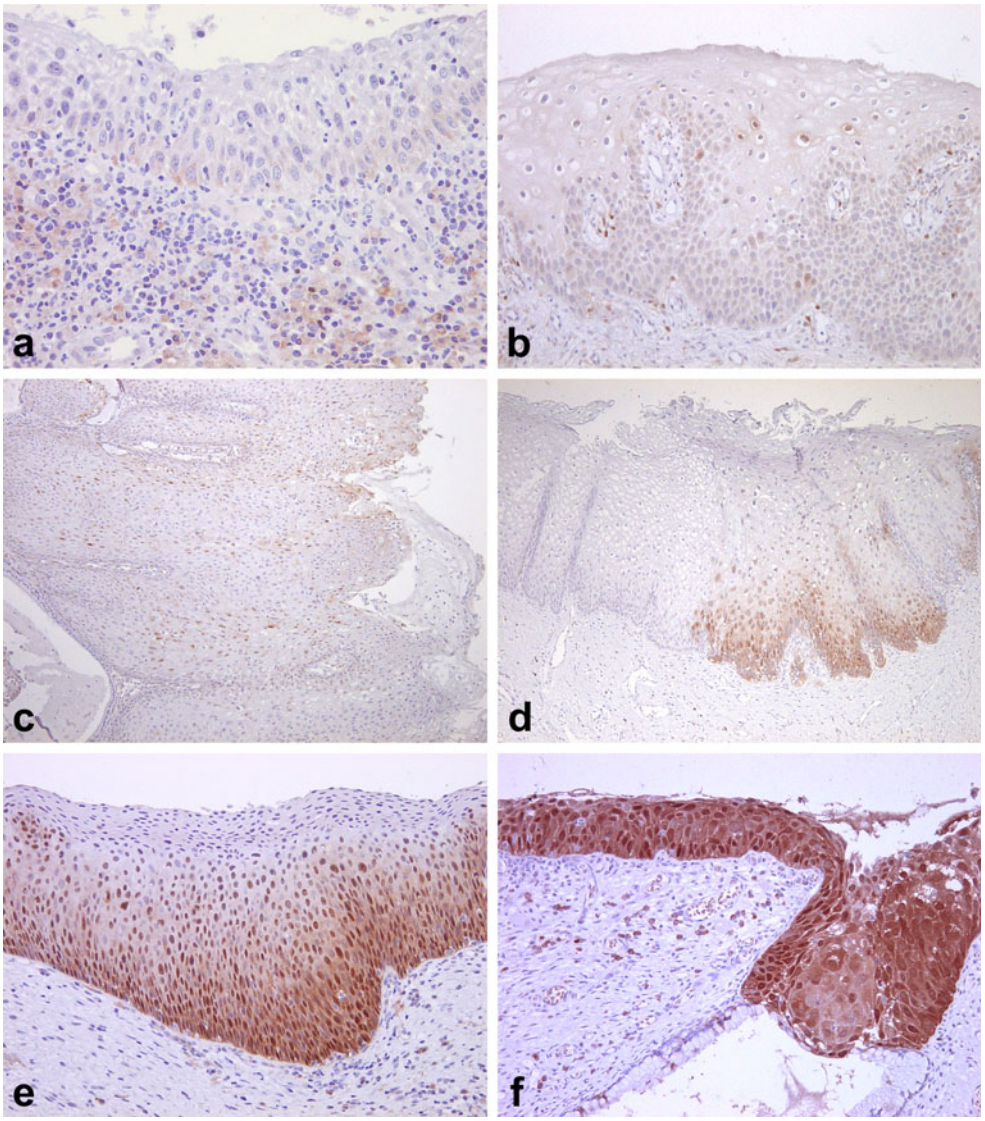

Fig. 2 Examples of p16^{INK4a} immunoprofiles differing between particular groups of lesions. **a** negativity in the metaplastic squamous epithelium with reactive changes (RC) (200×); **b** sporadic, weak, full-thickness positivity in the metaplastic squamous epithelium with koilocytosis (Kc) (200×); **c** focal, weak, full-thickness positivity in flat condyloma (Co) (100×); **d** sharp transition between the non-dysplastic squamous epithelium and CIN I with a diffuse, weak, lower half positivity (100×); **e** immunostaining extending into the upper half of the epithelium in CIN II interpreted as a diffuse, strong, full-thickness positivity (200×); **f** diffuse, strong, full-thickness positivity in CIN III (200×)

diffuse, lower half staining of any intensity correspond to CIN I. HSIL phenotype is clearly defined by a diffuse, strong/weak, full-thickness p16^{INK4a} positivity. Focal, strong, full-thickness p16^{INK4a} staining observed in one case of CIN III probably represent HSIL phenotype as well, as it was not seen in any other type of lesion except AIM.

Predictive Significance of p16^{INK4a} Immunoprofiles

The CIN I group showed the most heterogeneous p16^{INK4a} expression, with a total of six p16^{INK4a} immunoprofiles. Although the majority of CIN I lesions were characterized by the LSIL/HPV phenotype, some of them (5.7 %) with a diffuse, strong, full-thickness positivity displayed the HSIL phenotype. This finding raises the question of whether a heterogeneous spectrum of p16^{INK4a} immunoprofiles in the CIN I group reflects different precancerous potentials of particular lesions. It is well known that p16^{INK4a} expression correlates with the spectrum of HPV types involved in the pathogenesis of the lesion. Diffuse and strong p16^{INK4a} immunostaining was observed mostly in lesions associated with HR-HPV types, whereas sporadic, focal and weak staining or no expression were found in cases infected with low risk HPV [14–17]. In addition, more intense p16^{INK4a} expression was detected in lesions with HPV DNA integrated into the host genome [17].

Given that the level of p16^{INK4a} expression correlates with the grade of CIN as well as with the HPV profile and the HPV integration status, it is not surprising that the diffuse p16^{INK4a} staining was confirmed to be an adverse prognostic factor in CIN I lesions, where it was associated with a higher rate of progression [20] or a shorter interval for progression [21]. Alternatively, p16^{INK4a} negative or non-diffusely stained CIN I did not progress to HSIL [22]. Therefore, we believe that some of the CIN I cases in our series, especially those with the HSIL phenotype, are prone to progress to HSIL.

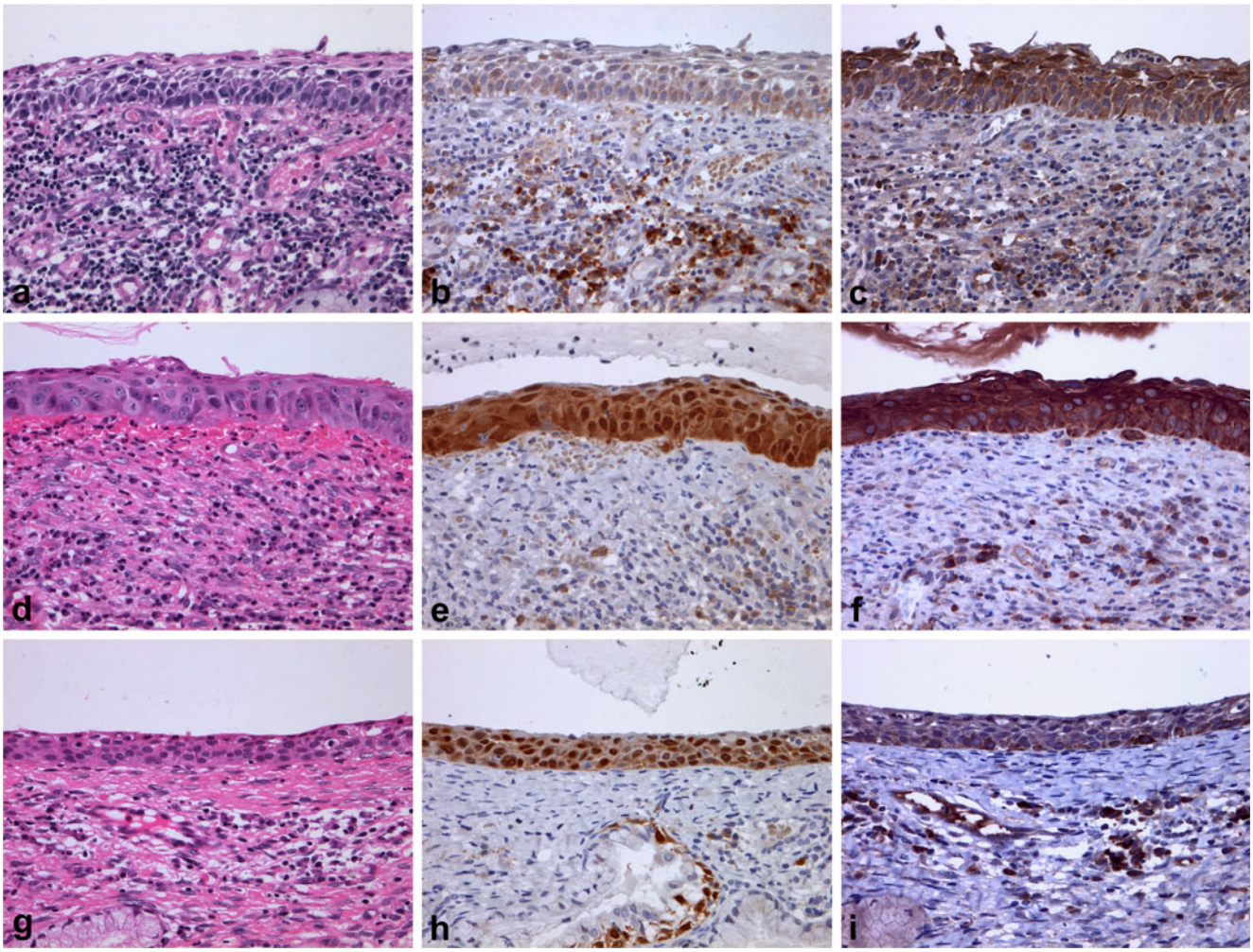

Fig. 3 Examples of AIM with various $p16^{INK4a}$ and CK 17 immunoprofiles (corresponding hematoxylin-eosin (HE) stained sections are shown). **a**, **b**, **c** AIM with the metaplastic phenotype (**a** HE, **b** $p16^{INK4a}$ negativity, **c** diffuse CK 17 positivity, 400×); **d**, **e**, **f** AIM with the HSIL phenotype (**d** HE, **e** diffuse, strong, full-thickness $p16^{INK4a}$ positivity, **f** diffuse CK 17 positivity, 400×); **g**, **h**, **i** AIM with the HSIL phenotype (**g** HE, **h** diffuse, strong, full-thickness $p16^{INK4a}$ positivity, **i** focal CK 17 positivity of single cells and clusters of cells in the basal zone of the epithelium, 400×)

Table 3 An overview of rates of CK 17 immunoexpression patterns in particular groups of AIM diversified according to their $p16^{INK4a}$ immunoprofiles and stratified into three basic clinically relevant phenotype groups (metaplastic, LSIL/HPV and HSIL)

$p16^{INK4a}$ immunoprofile	Phenotype	CK 17 immunoexpression	
		Diffuse	Focal
Negative	Metaplastic	10/10	–
Sporadic, weak, full-thickness	LSIL/HPV	2/2	–
Focal, weak, full-thickness	LSIL/HPV	9/9	–
Focal, strong, full-thickness	HSIL	–	2/2
Difuse, strong, full-thickness	HSIL	4/8	4/8

CK 17 cytokeratin 17, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *HPV* human papillomavirus

CIN II is also considered a heterogeneous category of lesions with a various tendency to regression [23]. This could be partially caused by a relatively low reproducibility of CIN II diagnosis when compared with CIN III [3]. However, we did not identify any significant difference between $p16^{INK4a}$ immunoprofiles of patients diagnosed with CIN II and CIN III. The CIN II group therefore seems to be consistent in our series. Importantly, the strong immunoexpression of $p16^{INK4a}$ in CIN II was shown to be associated with a persistence or even progression into CIN III [17].

Reclassification of the AIM group

The AIM group showed five $p16^{INK4a}$ immunoprofiles, making it the second most heterogeneous group in our series. The majority of AIM lesions (68.8 %) were $p16^{INK4a}$ positive and

showed HSIL, LSIL/HPV and metaplastic phenotypes in 34.4 %, 34.4 % and 31.2 % of the cases, respectively. These results suggest that approximately one third of the AIM cases in our study should be considered as a HSIL or a lesion with a potential to progress to HSIL, one third of the AIM group can be reclassified as LSIL or a manifestation of HPV infection and one third represents an immature squamous metaplasia. The immunohistochemical assessment of p16^{INK4a} expression was already shown to be beneficial in the estimation of the biologic behavior of AIM [6, 7, 13]. The proportion of lesions with p16^{INK4a} overexpression fluctuated in the interval 41–65 % [6, 13], with 19 % [6] to 65 % [13] subsequently reclassified as HSIL. These data were supplemented by HPV typing studies, which detected intermediate/HR-HPV types in up to 67 % of the AIM cases [5].

CK 17 represents another immunohistochemical marker which has been evaluated in AIM. Regauer et al. [13] reported the reciprocal immunoreactivity of p16^{INK4a} and CK 17 in immature squamous metaplasia and CIN III and suggested that these two antibodies should be used for the reclassification of AIM and that the term AIM should be withdrawn from the terminology. However, this observation was not confirmed by other studies which describe not only the immunoexpression of CK 17 in all grades of CIN, but also its correlation with the increasing grade of the lesion [11, 12]. In our series, all AIM cases with p16^{INK4a} immunoexpression consistent with metaplastic and LSIL/HPV phenotype displayed a diffuse pattern of CK 17 staining. A heterogeneous CK 17 immunoexpression was observed in AIM lesions with HSIL phenotype where focal CK 17 positivity prevailed (60.0 %) and the diffuse CK 17 staining was detected less frequently (40.0 %). Regauer et al. [13] described similar coexpression of p16^{INK4a} and CK 17 immunomarkers in 15 % of AIM cases and reclassified all these lesions as CIN III. We appreciate this opinion and recommend to prefer p16^{INK4a} as a more reliable marker until the role of CK 17 immunohistochemistry in the differential diagnosis between immature squamous metaplasia and CIN III will be clarified on a larger series of cases.

Conclusions

p16^{INK4a} immunohistochemistry based on the evaluation of the intensity and horizontal and vertical distribution of staining appears as a suitable supporting method for the classification of squamous lesions of the uterine cervix. Furthermore, it may also be used for the reclassification of categories with the heterogeneous p16^{INK4a} expression (especially AIM). We strongly encourage pathologists to use the p16^{INK4a} immunohistochemistry in these specific indications. On the other hand, the efficacy of CK 17 immunohistochemistry seems to be controversial for these purposes.

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Conflict of Interest The authors declare that they have no conflict of interest.

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