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Sec62/Ki67 and p16/Ki67 dual-staining immunocytochemistry in vulvar cytology for the identification of vulvar intraepithelial neoplasia and vulvar cancer: a pilot study

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Abstract

Purpose The aim of this study was to analyze the diagnostic performance of a newly established immunocytochemical dual-staining protocol for the simultaneous expression of *SEC62* and Ki67 in vulvar liquid-based cytology specimens for the identification of vulvar intraepithelial neoplasia (VIN) and vulvar cancer. In addition, we investigated the p16/Ki67 dual stain, which has already been established in cervical cytology.

Materials and methods For this pilot study, residual material from liquid-based cytology was collected retrospectively from 45 women. The presence of one or more double-immunoreactive cells was considered as a positive test result for Sec62/Ki67 and p16/Ki67 dual staining. The test results were correlated with the course of histology.

Results All cases of VIN and vulvar cancer were Sec62/Ki67 and p16/Ki67 dual-stain positive, and normal and low-grade squamous intraepithelial lesions were all negative. The sensitivity of cytology for VIN + cases was 100% (22/22), whereas punch biopsy classified one case of vulvar carcinoma as inflammation. All cases with high-intensity (grades 3 and 4) Sec62 staining in Sec62/Ki67-positive cases were carcinomas.

Conclusions The results of this study demonstrate that Sec62/Ki67 and p16 Ki67 dual-staining cytology could be a promising adjunctive diagnostic tool for VIN and squamous cell carcinoma, in addition to standard histology.

Keywords Sec62 · Ki67 · Immunocytochemistry · Vulvar cytology · Vulvar intraepithelial neoplasia · Vulvar cancer

Background

Vulvar cancer is the fourth most common gynecological malignancy in the United States after uterine, ovarian and cervical cancers [1]. Squamous cell carcinoma (SCC) is the most common histological subtype [2–4]. Other less common histologies include basal cell carcinoma, Paget's disease, adenocarcinoma and melanoma [4]. Buttmann-Schweiger et al. analyzed the data on vulvar carcinoma in eight population-based German cancer registries and found that the annual incidence of invasive cancer nearly doubled between 1999 and 2011 [3]. An increased age-standardized incidence rate of vulvar cancer in women of all ages was also found in other high-income countries (Canada, United States, Denmark, France, Iceland, Ireland, Sweden, Switzerland, Netherlands, United Kingdom, Australia and Japan) between 1988–1992 and 2003–2007 [5]. This increase in incidence is attributable to a significant increase in women aged < 60 years who were diagnosed with this form of cancer, which is partially explained by an increased HPV prevalence. The incidence of vulvar cancer is expected to increase in the future due to population growth and an aging population, but this increase should be slowed down by the introduction of the HPV vaccine [5].

Biopsy is the standard procedure for the investigation of suspected premalignant or malignant vulvar lesions [6]. Punch biopsy of the vulvar lesion should be taken at the peripheral edge of the tumor to enclose the underlying

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stroma and avoid the necrotic center of the tumor. The biopsy should provide reliable pathological samples. However, it has limitations. Tangential cutting or other artifacts can lead to misinterpretations; so even with this invasive method, one might overlook an invasive cancer [7]. Relying solely on biopsy results may lead to undertherapy of the patient [8].

The amplification of the 3q chromosomal region has been frequently detected in vulvar and cervical SCC [9–12]. The *SEC62* gene is encoded in chromosomal region 3q26 [13]. The amplification of chromosomal region 3q26 has been demonstrated in numerous human cancers, including cervical carcinoma, vulvar cancer, non-small cell lung cancer, esophageal cancer, ovarian carcinoma, and head and neck tumors [10, 12, 14–21].

In a study by Linxweiler et al., increasing *SEC62* amplification in dysplastic cervical lesions as well as increased cellular Sec62 protein levels were correlated with the severity of dysplasia in liquid-based cytology. Functional analyses showed an increased ability of HeLa cells to migrate after *SEC62* overexpression [12].

Ki-67, a nuclear antigen, can be detected by immunocytochemistry (ICC) in the G1, S, G2, and mitotic phases but is undetectable in resting (G0) cells. Therefore, Ki-67 ICC allows the identification of proliferating benign and malignant cells [22].

Using the combination of two biomarkers (p16 and Ki67) in a double-staining ICC protocol is widespread in cervical cytology but has not been established in vulvar cytology [23–25].

Therefore, the aim of our study was to clarify the value of vulvar LBC with a colposcopically targeted smear from the lesion. We also wanted to identify the extent to which vulvar cytology with Sec62/Ki67 and p16/Ki67 ICC could be used to detect malignant and premalignant vulvar lesions.

Materials and methods

Patients and LBC samples

Residual cytological material from ThinPrep (Hologic, Marlborough, Massachusetts, USA) LBC samples from 45 patients who underwent colposcopy was available. All patients gave written consent for the use of their cytologic samples according to the Declaration of Helsinki, and the study protocol was approved by the local ethical review board.

Vulvar cytology

The samples were collected with a Vulvar Sampler (Rovers Medical Devices, Oss, Netherlands) according to the manufacturer's instructions [26]. The lesions were brushed three times at the punctum maximum of the lesion. Slides were prepared with a T2000 processor (Hologic), Papanicolaou stained according to the standard protocol and subsequently assessed by two experienced cytotechnologists.

Based on the Bethesda System, the following cytological diagnoses were made: normal/vulvitis for benign cellular changes, LSIL—low-grade squamous intraepithelial lesion, and HSIL—high-grade squamous intraepithelial lesion, suspicious for SCC [26, 27].

Colposcopy, histology, p16/Ki67 dual stain

Colposcopic and histologic findings were reviewed. Colposcopy was performed according to the colposcopic nomenclature for vulvar lesions from the International Federation for Cervical Pathology and Colposcopy [28]. Except for 9 cases with normal or minor colposcopic findings, histology was performed (in 18 cases, target biopsy only; in 18 cases, target biopsy followed by complete excision). P16/Ki67 dual staining was performed in 27 cases with residual liquid-based-cytology (LBC) material after Sec62/Ki67 dual staining using a CINtec PLUS kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Sec62/Ki67 dual stain in vulvar cytology

To establish the Sec62/Ki62 dual stain in vulvar cytology, the Sec62 and Ki67 immunostains were individually tested with different antibody dilutions (1:400, 1:600, 1:800) as already described in cervical cytology (submitted). The Sec62 staining in the 1:400 dilution and the Ki67 staining in the 1:600 dilution were successful. This step was followed by a sequential dual-staining procedure in different orders (Sec62 followed by Ki67 and Ki67 followed by Sec62) and with different dilutions (1:400, 1:600, 1:800) in all possible combinations. Finally, simultaneous staining was tested with antibody cocktails with different dilutions of each antibody. In addition, individual steps of staining (fixation, unmasking, blocking) were performed with different methods to achieve the best possible results.

Slides were prepared with a T2000 processor (Hologic), ethanol fixed and dried overnight at room temperature. Heat-induced epitope retrieval (95 °C) was performed in retrieval buffer (Tris/EDTA-buffer solution pH 9.0—Roche, Basel, Switzerland) for 20 min, and nonspecific protein binding sites were blocked by incubation in 3% BSA (bovine serum albumin)-PBS (phosphate buffer solution) (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) for 30 min at room temperature. Subsequently, coating was carried out with the primary antibody cocktail (Ki67 (Dako Agilent Technologies, Santa Clara, California, USA) at 1:600 and Sec62 (Institute for Medical Biochemistry and Molecular

Biology—Saarland University Medical Center, Homburg, Germany) at 1:400 in 1% BSA-PBS solution) for 60 min at 37 °C. Slides were coated with the visualization reagents alkaline phosphatase (Roche, Basel, Switzerland), horseradish peroxidase (Roche), DAB (3,3′-diaminobenzidine) substrate-chromogen solution (Roche), and fast-red chromogen solution (Roche) using a CINtec PLUS kit (Roche) according to the manufacturer's instructions. This process led to red and brown staining at the Sec62 and Ki67 antigen sites, respectively. This step was followed by counterstaining with hematoxylin (alcohol free—Sigma Aldrich Chemie GmbH). The preparations underwent a 2-step mounting procedure, first with an aqueous mounting medium, followed by a mounting step with Entellan (Sigma Aldrich Chemie GmbH) [29].

Interpretation of the Sec62/Ki67 dual stain

Sec62/Ki67 dual staining was considered positive if double-immunoreactive squamous cells were present. We considered cells with cytoplasmic red (Sec62) and nuclear brown (Ki67) staining to be positive (Fig. 1). Cases without dual-positive squamous cells were considered negative.

P16/Ki67 dual stain in vulvar cytology

A CINtec PLUS kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Positivity was defined by cytoplasmic brown (p16) and nuclear red (Ki67) staining (Fig. 2).

Statistical analysis

For the statistical analysis, Chi-square test and Fisher's exact test were used with SPSS software, version 25 (IBM, Chicago, Illinois). P values of <0.05 were considered as statistically significant ($\alpha = 0.05$).

Results

This analysis included 45 cases. Cytological and colposcopic findings were categorized as "benign"—Normal/LSIL, "suspicious for VIN"—HSIL and "suspicious for malignancy"—SCC for statistical evaluation (Table 1). In 9 cases with normal colposcopy, no histology was available; in the remaining 36 cases, histology (only punch in 18 cases and punch and complete excision in 18 cases) was performed.

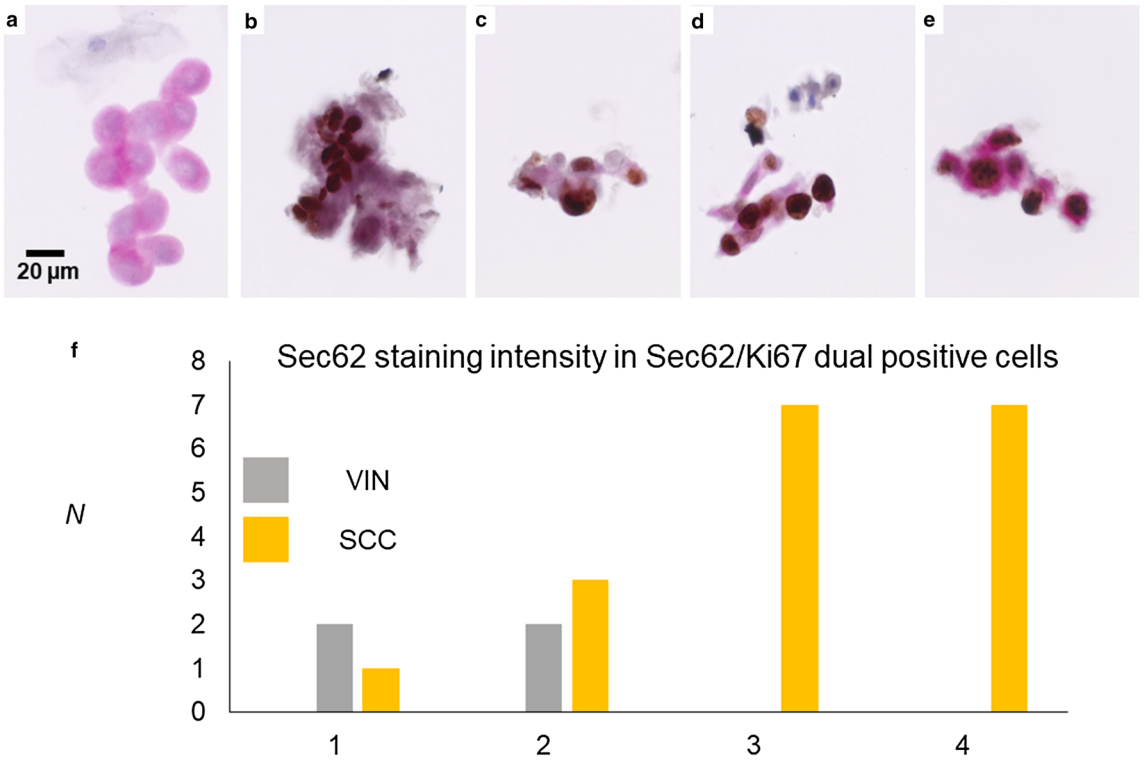

Fig. 1 Immunocytochemical features of Sec62/Ki67 expression in vulvar LBC specimens. **a** Cells negative for Sec62/Ki67 dual staining, as well as reactive squamous epithelial cells with high-intensity Sec62 staining without Ki67 positivity. Dual-stain-positive vulvar specimens: Sec62 immunostaining intensity grade 1 (**b**), 2 (**c**), 3 (**d**) and 4 (**e**). **f** Cases with VIN/SCC according to the Sec62 staining intensity. No normal/LSIL case showed Sec62/Ki67 positivity, and all SCC cases showed a high (grade 3 or 4) staining intensity

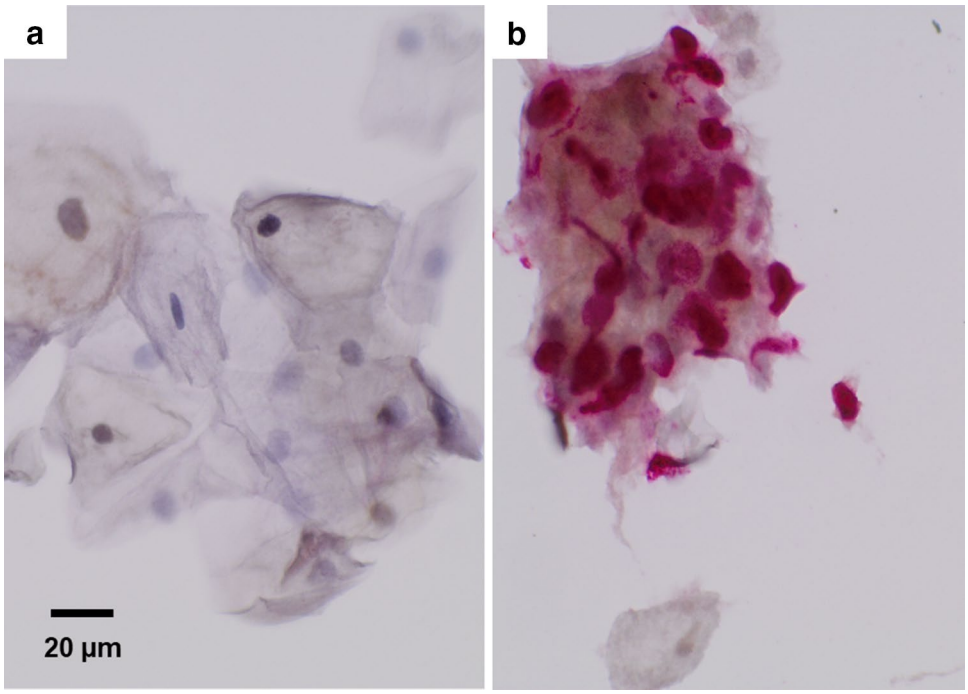

Fig. 2 p16/Ki67 immunocytochemistry in vulvar cytology. **a** Negative and **b** positive for the p16/K67 dual stain

Table 1 Categorization of cytological and colposcopic findings

Category	Cytological findings	$N=45$	Colposcopic findings		$N=45$
Normal/LSIL	Normal/vulvitis	19	Normal/inflammatory	Normal	10
				Eczema	5
	LSIL	3	Minor changes	Lichenification	4
				Wart	2
HSIL	HSIL	8	Major changes	Strong acetowhite epithelium	3
SCC	HSIL suspicious for invasion	5	Suspicion of malignancy	Ulceration	8
	SCC/malignancy	10		Gross neoplasm	4
				Atypical vessels	1
				Bleeding	2
				Exophytic	2
				Hyperkeratosis	4

VIN vulvar intraepithelial neoplasia, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *SCC* squamous cell cancer

Sec62/Ki67 dual staining and p16/Ki67 dual staining of liquid-based vulvar cytology specimens

Of the 45 patients, all (100%) vulvar intraepithelial neoplasia (VIN)/SCC cases showed Sec62/Ki67 positivity, while all (100%) low-grade lesions and normal cells were negative (Table 2). P16/Ki67 was also positive in all VIN/SCC cases and negative in normal cells (Table 2). In Sec62/Ki67 positive cases, a different Sec62 staining intensity could be observed (Fig. 1). Every case with a Sec62 staining intensity of 3–4 was revealed as a malignancy; four cases with malignancy showed a lower (1–2) staining intensity, and none of the SCC cases was negative with the Sec62/Ki67 dual stain (Table 2). Detailed information on the histological, cytological findings and staining results are delineated in Table 2.

To enable a comparison, despite the small sample size, we calculated the sensitivity and specificity of different diagnostic methods for detecting VIN and SCC (Table 3). Since all cases with normal findings or low-grade lesions of the vulva were Sec62/Ki67 and p16/Ki67 negative and all VIN + findings were positive, we were able to calculate a 100% sensitivity and specificity for these methods. One case was overrated by cytology, and punch biopsy missed a case of VIN; consequently, cytology had a specificity of 95.7%,

Table 2 Clinical and histological correlation of cytological and immunocytochemical findings

Diagnostic method	*N*	Result	Normal[a]/LSIL *n* (%)	HSIL *n* (%)	SCC *n* (%)
			23	4	18
Cytology	45	Normal/LSIL	22 (95.7)	0 (0)	0 (0)
		Suspicious for HSIL	1 (4.3)	2 (50)	5 (27.8)
		Suspicious for SCC	0 (0)	2 (50)	13 (72.2)
Sec62/Ki67 dual stain	45	Negative	23 (100)	0 (0)	0 (0)
		Positive	0 (0)	4 (100)	18 (100)
		Low (Sec62 intensity 1–2)	0 (0)	4 (100)	4 (22.2)
		High (Sec62 intensity 3–4)	0 (0)	0 (0)	14 (77.8)
Punch biopsy	36		14	4	18
		Normal/LSIL	14 (100)	0 (0)	1 (5.6)
		HSIL	0 (0)	4 (100)	3 (16.6)
		SCC	0 (0)	0 (0)	14 (77.8)
p16/Ki67 dual stain	27		10	3	14
		Negative	10 (100)	0 (0)	0 (0)
		Positive	0 (0)	3 (100)	14 (100)

LSIL low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *SCC* squamous cell cancer

[a]In 9 cases with normal colposcopic findings, no histology was performed

and punch biopsy had a sensitivity of 95.5%. In the detection of SCC, the sensitivity of the Sec62/Ki67 and p16/Ki67 double-staining methods was 100%, but since all VIN cases were positive, the specificity was lower (85.2% and 76.9%). All cases with highly intensive Sec62 staining were carcinomas, leading to the highest specificity for the identification of malignancy among the methods studied.

Discussion

This is the first study that has investigated Sec62/Ki67 dual-staining and p16/Ki67 dual-staining ICC in vulvar cytology. The data from this study suggest that both Sec62/Ki67 dual staining and p16/Ki67 dual staining are possibly useful for cytologists for the detection of VIN + with a comparably high sensitivity and specificity in liquid-based cytological specimens of the vulva (Table 3.). In detecting malignancy, punch biopsy showed results comparable with those of cytology and with the Sec62/Ki67 staining intensity. The limitations of this study are its retrospective manner, the comparably small number of cases and the unknown HPV status.

Since Sec62 is a cytoplasmic marker, atypical cells without a cytoplasm (so-called naked or bare nuclei) cannot be dual-stain positive, even if they are morphologically severely dysplastic. The same is true for the typical anucleate cells with severe cytoplasmic alterations found in vulvar cancer. Normally, however, the typical cellular phenomena of SCC and VIN are mixed; so, this limitation may apply to single cells but probably not to the entire slide. A further limitation of the Sec62/Ki67 dual stain is that it can be false positive in reactive glandular cells. As vulvar glandular cells or benign glandular tumors such as papillary hidradenoma are rarely found, this restriction is not very relevant [30].

Linxweiler et al. detected a *SEC62* gene amplification with FISH (fluorescence in situ hybridization) as well as increasing *SEC62* expression with immunofluorescence cytology in dysplastic cervical lesions in LBC, depending on the severity of the dysplasia [12]. Sec62 presumably represents a marker for epithelial–mesenchymal transition, and high expression of *SEC62* indicates the malignant potential of the cells. These findings correlate with studies on amplification of the 3q chromosomal region, which has been detected predominantly in HPV-positive vulvar and cervical cancers [9–12, 14].

In earlier studies, Ki67 expression was shown in normal tissue or in conjunction with reactive changes in the parabasal cells or in the lower cell layers of the epidermis. In uVIN (usual-type vulvar intraepithelial neoplasia), there was diffuse positivity of all epithelial cell layers, and in dVIN (differentiated-type vulvar intraepithelial neoplasia), only focal positivity was found, thereby enabling the differentiation of VIN from normal vulvar epithelium [31, 32].

The combination of a proliferation marker such as Ki67 and a migration marker such as Sec62 may be helpful for the identification of cells with malignant potential. We expected that cells with a malignant potential, i.e., high-grade precancerous lesions and carcinomas, would show dual reactivity.

Our findings support this hypothesis in that VIN and SCC cases were Sec62/Ki67 positive, and most SCCs even

Table 3 Sensitivity and specificity of vulvar cytology, Sec62/Ki67 dual staining, and p16/Ki67 dual staining for the detection of VIN and vulvar cancer

	Normal/LSIL (*n*)	VIN/SCC (*n*)	Sensitivity (%)	Specificity (%)		Normal/LSIL/HSIL (*n*)	SCC (*n*)	Sensitivity %	Specificity %
Cytology									
N = 45	23	22	100	95.7	*N* = 45	27	18	72.20	92.60
Normal/LSIL	22	0			Normal/LSIL/HSIL	25	5		
VIN/SCC	1	22			SCC	2	13		
Punch biopsy									
N = 36	14	22	95.5	100	*N* = 36	18	18		
Normal/LSIL	14	1			Normal/LSIL/HSIL	14	1	94.4	77.8
VIN/SCC	0	21			SCC	4	17		
Sec62/Ki67									
N = 45	23	22	100	100	*N* = 45	27	18	100.0	85.2
Negative	23	0			Negative	23	0		
Positive	0	22			Positive	4	18		
Sec62 high intensity									
N = 22	0	22			*N* = 22	4	18		
Low	0	8	[a]	[a]	Low	4	4	77.8	100.0
High	0	14			High	0	14		
p16/Ki67									
N = 27	10	17			*N* = 27	13	14		
Negative	10	0	100	100	Negative	10	0	100.0	76.9
Positive	0	17			Positive	3	14		

LSIL low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *VIN* vulvar epithelial neoplasia, *SCC* squamous cell cancer

[a]Since double staining was positive for all cases with VIN/SCC and negative for all normal/LSIL cases, the sensitivity and specificity for high-intensity dual staining could not be calculated

showed stronger staining with Sec62 than VIN did; moreover, low-grade lesions and reactive changes were nonreactive. Of the total of 18 SCC cases, 14 showed a Sec62 staining intensity grade of 3 or 4. No VINs showed a staining intensity higher than grade 2. These results suggest that in addition to Sec62/Ki67 positivity, Sec62 staining intensity can also be useful in the recognition of SCC and could be used in distinguishing VIN from SCC.

The simultaneous detection of p16 and Ki67 overexpression has been investigated in cervical cytology in several studies demonstrating that the p16/Ki67 dual stain is a useful tool for the detection of cervical intraepithelial neoplasia (CIN) lesions in cervical and oropharyngeal cytology [23–25, 34, 35]. The detection of p16/Ki67 coexpression facilitates the identification of cells with a deregulated cell cycle in cervical cytology samples, regardless of the morphology-based interpretation parameters. The presence of 1 or more double-immunoreactive cells may be an indicator of underlying CIN [24, 25, 33, 34]. In our small collective, all VIN/SCC samples were p16/Ki67 positive. This finding suggests that p16/Ki67 may also be a useful tool in vulvar cytology for unclear cytologic and colposcopic findings in the detection of VIN and SCC.

Cytology has proven to be a reliable, patient-friendly method for the diagnosis of cervical (pre)malignant diseases and has led to a significant decrease in cervical carcinoma incidence through screening programs [36]. The accuracy of cytology depends largely on the presence of a sufficient number and quality of cells and the ability to recognize cellular and nuclear atypia. The role of cytology in the diagnosis of vulvar pathologies is debated in the literature. Different techniques have been investigated, with different, sometimes unsatisfactory results [26, 27, 37–42].

Despite the small number of cases and different methods for sampling and processing, our accuracy in cytology is comparable to that of Dennerstein et al., Jimenez et al., Levine et al. and Van den Einden et al. [26, 27, 38, 42]. Scalpel scrapings and conventional cytology from the suspicious lesions lead to high detection rates for SCC. Dennerstein et al. detected cells with typical signs of malignancy in 12

out of 12 cases (100%), Jimenez-Ayala et al. also achieved a high sensitivity and specificity for detecting malignancy (98.21% and 94.82%) [27, 38].

Levine et al. used a cytobrush (rotated 20×) for targeted sampling of vulvar lesions [42]. The exfoliated cells were extracted and concentrated in several steps, and 3 slides were prepared with the cytospin method. With this complex procedure, they were able to achieve 100% sensitivity for the detection of VIN and AIN [42]. Van den Einden et al. used a Vulvar Sampler (Rovers Medical Devices, Oss, Netherlands) to collect 65 targeted smears after washing of the circumscribed lesions in 37 patients with LS and suspicious lesions for VIN or SCC with physiological NaCl solution for ThinPrep cytology [26]. Twenty-six percent of the slides were inadequate because of poor cellularity; 28 of 29 (97%) VIN/SCC lesions were found to be suspicious or uncertain for "(pre)malignancy". Cytology demonstrated 100% sensitivity and a negative predictive value of 100% in the case of malignancy. Specificity was 50% for both premalignancy and malignancy [26]. Nauth and Schilke studied the vulvar cytology of 464 women. The samples were taken with a moist cotton swab. Of the 111 histologically proven malignant lesions, only 50% showed tumor cells in the cytological preparations [39]. In a later study, the same authors obtained a higher detection rate (66%) for malignant lesions [40]. Bae-Jump et al. retrospectively examined vulvar cytology specimens (each collected with a spatula) from 50 patients (43 conventional and 7 ThinPrep LBC), along with existing histology findings. Only 7 out of 22 cases (32%) with VIN or SCC were found to be suspicious for VIN or SCC with the cytology method [41]. An explanation for these different results could be the different sampling methods [43]. In our opinion, it is important to carry out the cell collections separately for each lesion so that the severity of the lesions can be individually assessed.

We only investigated cases where there was enough residual material left over to perform ICC. Thus, cases with an insufficient number of cells were naturally excluded. However, we were able to show that in cases with a representative sample, recognition and differentiation of benign findings from VIN and SCC are possible (Table 2).

Colposcopy or simple visual inspection of the vulva is essential to make a diagnosis. For proper sampling for cytology and histology, a certain level of experience is necessary. The general recommendation to remove specimens at the border of the lesion may result in missing a central invasion. The histological detection of a microinvasion is also challenging, and even more difficult with small, office punch biopsies [7, 8, 44]. Preti et al. studied 216 cases with complete VIN excision after previous punch biopsy. In 24 cases (11%), SCC was found in the surgical specimens. The researchers identified patient age, lesion size (diameter ≥ 20 mm), clitoral involvement, and nodular lesions as risk factors of unrecognized invasive cancer in patients with VIN lesions [8]. If stromal invasion cannot be detected, it may result in undertreatment of the patient, which in turn can negatively influence the prognosis. Such cases can undermine physicians' confidence in conservative VIN treatments and could lead to unnecessarily radical surgery [8].

Conclusions

Our results suggest that for detecting invasive lesions of the vulva in early-stage cancer, the sensitivity of cytology is comparable with that of punch biopsy and a high Sec62 staining intensity (intensity grade 3–4).

LBC techniques with and without Sec62/Ki67 and p16/Ki67 dual staining could be valuable complementary methods for the diagnosis of vulvar diseases. Because of the lack of prospective studies for evaluating the safety of cytology ± ICC in routine diagnostics, the standard method of clarification should remain histology.

Targeted vulvar cytology with or without ICC as a less invasive diagnostic tool could improve patient comfort, especially in patients with chronic vulvar disease, and may substantiate a biopsy indication or even indication for complete excision without a prior biopsy.

In our opinion, if cytological signs of malignancy or high-intensity Sec62 staining in Sec62/Ki67 dual-positive cells are present, even if a punch biopsy results in a VIN, complete excision of the lesion should be performed instead of ablative procedures.

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Author contributions FZT: conceptualization, data curation, methodology, project administration, validation, writing the original draft, and review and editing. JCR: review and editing. FB: visualization, review and editing. IJ-B: review and editing, E-FS: review and editing, RMB: review and editing. G-PB: review and editing. BS: review and editing. ML: supervision, methodology, project administration, validation, review and editing.

Compliance with ethical standards

Conflict of interest Ferenc Zoltan Takacs and Maximilian Linxweiler have a pending patent application. Patent applicant: University of Saarland. Names of inventors: Ferenc Zoltan Takacs, Maximilian Linxweiler. Application number: LU100824. All other authors declare no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of Saarland (No:

183/17). Written informed consent was obtained from all patients prior to inclusion.

Informed consent The study was approved by the Ethics Committee of Saarland (No: 183/17). Written informed consent was obtained from all patients prior to inclusion.

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