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Sec62/Ki67 dual staining in cervical cytology specimens: a new marker for high-grade dysplasia

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Abstract

Purpose In the previous studies, we demonstrated that Sec62 is essential for tumor cell migration, epithelial-to-mesenchymal transition, and intracellular stress tolerance. An increase in Sec62 expression correlated with an increase in cervical dysplasia severity in liquid-based cytology specimens. Ki67 is an established proliferation marker. Thus, in this study, we examined a method of Sec62/Ki67 dual staining for the detection of high-grade dysplasia and cancer in cervical liquid-based cytology specimens.

Methods Sec62/Ki67 dual staining was performed on 100 cervical liquid-based cytology specimens. The staining results were correlated with cytological, immunocytological (p16/Ki67), colposcopic, and histological findings.

Results All 56 ($n=56$, 100%) cases of cervical intraepithelial neoplasia grade 3 and cervical cancer (CIN3+ lesions) were positive for Sec62/Ki67 staining, while low-grade lesions and normal cells were negative. Sec62/Ki67 staining was highly sensitive and specific for the detection of CIN2+ and CIN3+ lesions (94.37%; 100% and 100%; 84.09%, respectively).

Conclusions Sec62/Ki67 dual-staining immunocytochemistry is a promising cytological tool for interpreting high-grade squamous lesions in cytological specimens and for assessing the risk of progression to cancer.

Keywords Cervical cancer · Cervical intraepithelial neoplasia · Cytology · Dual staining · Immunocytochemistry · Sec62

Background

Cervical cancer is the fourth most common cancer diagnosed and the fourth leading cause of cancer-related death, with approximately 527,600 new cases and 265,700 deaths occurring worldwide in 2012 [1]. Cervical cancer was the second most common cancer among women in the 1970s, but because of the improved cancer care, mortality has fallen drastically in developed countries [1, 2]. However, in developing regions without cervical screening programs, the incidence remains high [3].

A systematic review by Nanda et al. found the sensitivity of the Papanicolaou (Pap) smear to be 51% (range 30–87%) and the specificity to be 98% (range 86–100%) for detecting the cervical intraepithelial neoplasia grade 2 and above (CIN2+) [4]. Since the sensitivity and specificity of cervical cytology varies widely, numerous biomarkers for squamous epithelial lesions have been investigated and established [4–10].

An immunocytochemical assay targeting topoisomerase II-α (Top2a) and minichromosome maintenance protein 2 (MCM2) detects high-grade squamous intraepithelial lesions (HSILs) with high sensitivity (100%) and specificity (76.7%–87.5%) in cytology specimens [9, 10].

The detection of p16/Ki67 coexpression facilitates the identification of cells with a deregulated cell cycle in cervical cytology samples regardless of morphology-based interpretation parameters. The presence of one or more double-immunoreactive cells may be an indicator of underlying cervical intraepithelial neoplasia (CIN) [6–8, 11]. Schmidt et al. investigated p16/Ki67 dual staining in liquid-based cytology (LBC) samples [6]. In that study, p16/Ki67 dual

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staining was positive for CIN2+ lesions in 92.2% of the cases and for low-grade squamous intraepithelial lesions (LSILs) in 52.5% of the cases; in addition to similar sensitivity, p16/Ki67 dual staining had higher specificity for the detection of these pathologies than high-risk human papillomavirus (HPV) testing [6].

The amplification of the chromosome 3q region has been detected in cervical carcinoma in several studies [12–14]. Wright et al. have investigated 3q amplification with fluorescence in situ hybridization (FISH) in LBC specimens and were able to achieve a high sensitivity and specificity for the detection of CIN2–3 in an automated setting, indicating that an amplification of the 3q region represents a critical event during the progression from low-to high-grade cervical dysplasia and finally to the development of cervical cancer [15].

The Sec62 gene is encoded in the chromosomal region 3q26, and its amplification 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 [12, 14, 16–23].

A systematic study of the 3q26 region by gain-and loss-of-function studies identified Sec62/TLOC1 as a "tumor driver gene". The same group investigated the effect of RNA interference-mediated Sec62 knockout on cell proliferation in 16 different human cell lines and found that cell lines exhibiting 3q26 amplification rely on Sec62 for proliferation [24].

Additionally, high Sec62 expression levels were linked to an increased metastatic potential in human tumor cells, since they were correlated with the occurrence of lymph node metastases both in head and neck cancer and in non-small cell lung cancer [21, 22]. These results were strengthened by those of cell culture experiments demonstrating that a variety of human cancer cells rely on sufficient Sec62 levels for migration and invasion as the molecular basis of metastasis [14, 21, 22, 25, 26]. Accordingly, high Sec62 expression levels are a significant predictor of poor clinical outcome both in head and neck cancer and in non-small cell lung cancer, probably due to the correlation with higher metastatic potential [22, 26].

In our previous study, we detected a gradual increase in Sec62 gene amplification by FISH along with an increase in Sec62 expression by immunofluorescence cytology that depended on the severity of the dysplasia in dysplastic cervical lesions in LBC specimens [14].

One advantage of immunocytochemical methods over FISH is the possibility of co-evaluating the sample morphology during the evaluation; staining can be assessed under a light microscope without the need for complex equipment.

Our hypothesis is that the cervical cells with concurrent capabilities for migration (Sec62 positivity) and proliferation (Ki67 positivity) are cells with potential for the development of cervical cancer. The aim of the study was to establish a Sec62/Ki67 dual-staining method and to evaluate the value of Sec62/Ki67 dual staining as a biomarker for high-grade squamous dysplasia and early invasive cancer in LBC specimens.

Methods

Patients and tissue samples

Residual cytological material from 100 ThinPrep (Hologic, Marlborough, Massachusetts) Pap test LBC specimens from patients who underwent colposcopy was available. Except for two negative intraepithelial lesion or malignancy (NILM) cases, histology was performed for all cases (63 excisions and 35 targeted biopsies). The p16/Ki67 dual-staining results were known for all cases.

All patients provided written consent for the use of their tissue samples, according to the Declaration of Helsinki, and the study protocol was approved by the local ethics board.

Sec62/Ki67 dual staining

To establish the protocol for Sec62/Ki67 dual staining, Sec62 and Ki67 immunostains were individually tested at different antibody dilutions (1:400, 1:600, and 1:800). Sec62 staining at a 1:400 dilution and Ki67 staining at a 1:600 dilution were successful. The dilution testing was followed by testing the sequential dual-staining procedure in all possible combinations of the tested antibody application orders (Sec62 followed by Ki67 and Ki67 followed by Sec62) and antibody dilutions (1:400, 1:600, and 1:800). Finally, simultaneous dual staining was tested with antibody cocktails containing different dilutions of each antibody. In addition, the individual steps of the staining protocol (fixation, unmasking, and blocking) were performed with different methods to achieve the best possible results.

Slides were prepared with a T2000 processor (Hologic, Marlborough, Massachusetts), fixed in ethanol and dried overnight at room temperature. Heat-induced epitope retrieval (95 °C) was performed in retrieval buffer (Tris/EDTA buffer, pH 9.0—Roche, Basel, Switzerland) for 20 min, and nonspecific protein-binding sites were blocked by incubation in 3% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) (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) at 1:600 and Sec62 (Institute for Medical Biochemistry and Molecular Biology–Saarland University Medical Center, Homburg, Germany) at 1:400 in a 1% BSA-PBS solution) for 60 min at 37 °C. Slides were coated with the visualization reagents such as alkaline phosphatase

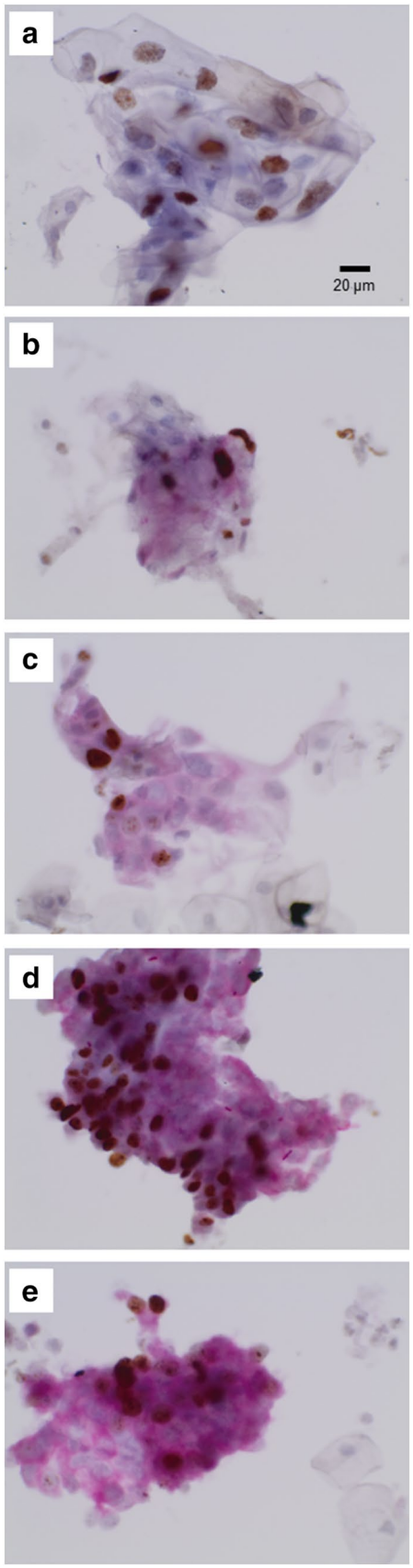

Fig. 1 Immunocytochemical features of Sec62/Ki67 expression in cervical LBC specimens. **a** Negative Sec62/Ki67 dual staining and **b** dual-stain-positive specimens with a Sec62 immunostaining intensity of grade 1, **c** grade 2, **d** grade 3, and **e** grade 4

(Roche, Basel, Switzerland) and horseradish peroxidase (Roche, Basel, Switzerland), followed by a 3,3′-diaminobenzidine (DAB) substrate chromogen solution (Roche, Basel, Switzerland), and finally a fast red chromogen solution (Roche, Basel, Switzerland) using a CINtec PLUS kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. This protocol 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, Taufkirchen, Germany). A two-step mounting procedure was performed by first using an aqueous mounting medium followed by Entellan (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Interpretation of the Sec62/Ki67 dual-staining results

Sec62/Ki67 dual staining was considered positive if double-immunoreactive squamous cells were present; cells with cytoplasmic red (Sec62) and nuclear brown (Ki67) staining were considered positive (Fig. 1). Cases without dual-positive squamous cells or those for which dual staining occurred only in glandular cells were considered negative.

Statistical analysis

For statistical analysis, a *t* test (two-sided) was used, and analyzes were performed with SPSS software, version 20 (IBM, Chicago, Illinois). $P < 0.05$ was considered statistically significant ($\alpha = 0.05$).

Results

Sec62/Ki67 dual staining in liquid-based cytology specimens

Figure 1 shows the features of Sec62/Ki67 dual staining. Only double-immunoreactive squamous epithelial cells were considered positive. We scored the intensity of the cytoplasmic Sec62 staining on a scale of 0–4.

Study population

This analysis included a total of 100 cases with available CINtec PLUS immunocytochemical results and histology results (except for two NILM cases and one atypical glandular cells not otherwise specified (AGC-NOS) case). All

56 (100%) CIN3+ cases were positive for Sec62/Ki67 dual staining, while cases involving low-grade lesions and normal cells were negative. Furthermore, p16/Ki67 dual staining was positive in all CIN3+ cases but was negative in normal cells. Two of 12 (16.7%) LSIL samples were p16/Ki67-positive (Table 1). Detailed information on the cytological diagnoses and staining results is presented in Table 1.

Since Sec62/Ki67 dual staining can be positive in reactive, proliferative endocervical glands, the assessment of cell morphology is indispensable for the evaluation of Sec62/Ki67 dual staining to prevent false-positive findings due to reactive endocervical cells. As Sec62 is a cytoplasmic marker, atypical cells without cytoplasm, which have so-called naked or bare nuclei, cannot be dual-stain-positive, even if they are severely dysplastic in terms of morphology. In our earlier study, we observed an increasing level of Sec62 expression with an increasing severity of dysplasia in LBC samples [14]. In dual-stain-positive samples in our current study, we found high Sec62 reactivity in CIN3+ cases and even higher Sec62 reactivity in SCC cases but no correlation with the degree of dysplasia. However, only SCC cases displayed grade 4 Sec62 staining intensity in dual-stain-positive atypical cells (Fig. 2).

Sensitivity and specificity of the dual-staining immunocytochemistry (ICC) (Sec62/Ki67, p16/Ki67)

The sensitivities, specificities, and predictive values for Sec62/Ki67 dual staining and p16/Ki67 dual staining were calculated for CIN2+ (comprising CIN2, CIN3, and SCC) and CIN3+ (comprising CIN3 and SCC) lesions and are listed in Table 2. The sensitivity of Sec62/Ki67 dual staining was 94.37% for detecting the underlying CIN2+ lesions and 100% for detecting the CIN3+ lesions. The corresponding specificities were 100% for CIN2+ lesions and 84.09% for CIN3+ lesions. p16/Ki67 dual staining demonstrated similar sensitivities for detecting CIN2+ (95.52%) and CIN3+ (100%) lesions. In terms of specificity, p16/Ki67 dual staining had lower specificity (CIN2+ 93.94%; CIN3+ 77.27%) than the Sec62/Ki67 dual staining, probably due to the positivity of LSIL cases. The positive predictive value (PPV) and negative predictive value (NPV) of Sec62/Ki67 cytology for detecting the underlying CIN2+/CIN3+ lesions were 100%/88.89% (PPV) and 89.20%/100% (NPV), and these values were 96.97%/84.85% (PPV) and 91.18%/100% (NPV) for p16/Ki67 dual staining.

Discussion

This study is the first to investigate Sec62/Ki67 dual staining. The data from this study suggest that both the p16/Ki67 and Sec62/Ki67 dual-staining ICC methods are useful for cytologists when interpreting high-grade squamous epithelial lesions in cytological specimens. Both immunostains are comparably sensitive for the detection of high-grade dysplasia in LBC samples.

Previous p16 single-staining ICC protocols required the interpretation of immunoreactive cell morphology to distinguish between p16-positive cells exhibiting dysplasia and cervical cells that occasionally overexpress p16 for physiological reasons, such as metaplastic squamous cells or endocervical cells [27]. We observed the same findings for Sec62 expression in reactive glandular cells. Sec62/Ki67 dual staining, however, was never falsely positive in the squamous epithelium. Unfortunately, Sec62/Ki67 dual staining is often positive in glandular cells. During the evaluation of the confusion surrounding “true positive” cells, morphologically conspicuous cells demonstrating “false positive” reactive changes can be a source of error. Therefore, the interpretation of the results requires special attention so that the staining is evaluated only in atypical squamous cells, whereas nonspecific glandular cells are ignored [28, 29]. The results of immunocytochemical studies from different research groups often vary greatly. This variation could probably explain the methodological difficulties inherent to ICC. The advantage of our manual staining approach was that we could modify the individual steps during the method development process to achieve an optimal result.

We found a correlation between Sec62/Ki67 positivity and CIN2+ lesions. This finding confirms our initial hypothesis that cells with a capacity for migration (Sec62 positivity) and proliferation (Ki67 positivity) are present in high-grade lesions which are known to have the potential to develop into cervical carcinoma. The isolated Ki67 positivity in reactive changes and LSIL cases is consistent with the observations of Konishi et al. and Dunton et al., who were able to detect Ki67 positivity in the basal, parabasal, and intermediate cells of condyloma and CIN lesions [30, 31].

Although we realized that the sample size is small, we calculated the accuracy of the Sec62/Ki67 and p16/Ki67 dual-staining cytology methods. Sec62/Ki67 dual staining had a higher specificity for CIN3+ lesions than the p16/Ki67 dual staining (Table 3). Because spontaneous regression can occur even in cases of untreated CIN3+ lesions, ideally, only those patients in whom cervical carcinoma would develop if surgery was not performed should undergo surgery [32]. This limitation would significantly reduce both the number of operations performed and the incidence of associated complications (miscarriage and premature birth). A recent study by Habbema et al. estimated the dangers of cervical cancer screening strategies in the US. In 2007, 36 million Pap smears showed 2.3 million abnormal results, leading to 1.5 million biopsies and 300,000 treatments due to precancerous lesions, which in turn were estimated to lead to 5000 preterm births [33]. The development of new biomarkers

Table 1 Sample characteristics according to Sec62/Ki67 and p16/Ki67 dual-staining results

Characteristics	No. of patients		Sec62/Ki67 dual staining				*P* value	Characteristics	No. of patients		p16/Ki67 dual staining				*P* value
	n = 100	%	negative		positive				*n* = 100	%	negative		positive		
			n = 37 (37%)	%	*n* = 63 (63%)	%					*n* = 34 (34%)	%	*n* = 66 (66%)	%	
Age (mean)	100	100	37.14 (SD 13.48)		35.46 (SD 8.9)		0.456[a]	Age			37.1 (SD 13.9)		35.6 (SD 8.9)		0.502[a]
Cytology								Cytology							
NILM	7	7	7	18.9	0	0		NILM	7	7	7	20.6	0	0	
AGC-NOS	1	1	1	2.7	0	0		AGC-NOS	1	1	1	2.9	0	0	
ASC-US	4	4	4	10.8	0	0		ASC-US	4	4	4	11.8	0	0	
ASC-H	17	17	4	10.8	13	20.6		ASC-H	17	17	4	11.8	13	19.7	
LSIL	12	12	12	32.4	0	0		LSIL	12	12	10	29.4	2	3	
HSIL	51	51	9	24.4	42	66.7		HSIL	51	51	8	23.5	43	65.2	
HSIL-suspicious for invasion	6	6	0	0	6	9.5		HSIL-suspicious for invasion	6	6	0	0	6	9.1	
SCC	2	2	0	0	2	3.2		SCC	2	2	0	0	2	3	
Dysplasia grade								Dysplasia grade							
No dysplasia	12	12	12	32.4	0	0		No dysplasia	13	13	13	35.3	0	0	
LSIL	21	21	21	56.8	0	0		LSIL	21	21	19	55.9	2	3	
HSIL (CIN2)	11	11	4	10.8	7	11.1		HSIL (CIN2)	11	11	3	8.8	8	12.1	
HSIL (CIN3)	51	51	0	0	51	81		HSIL (CIN3)	51	51	0	0	51	77.3	
SCC	5	5	0	0	5	7.9		SCC	5	5	0	0	5	7.6	

NILM negative for intraepithelial lesion or malignancy, *AGC-NOS* atypical glandular cells not otherwise specified, *ASC-US* atypical squamous cells of undetermined significance, *LSIL* low-grade squamous intraepithelial lesion, *ASC-H* atypical squamous cells, cannot exclude HSIL, *HSIL* high-grade squamous intraepithelial lesion, *SCC* squamous cell carcinoma

[a] *t* test

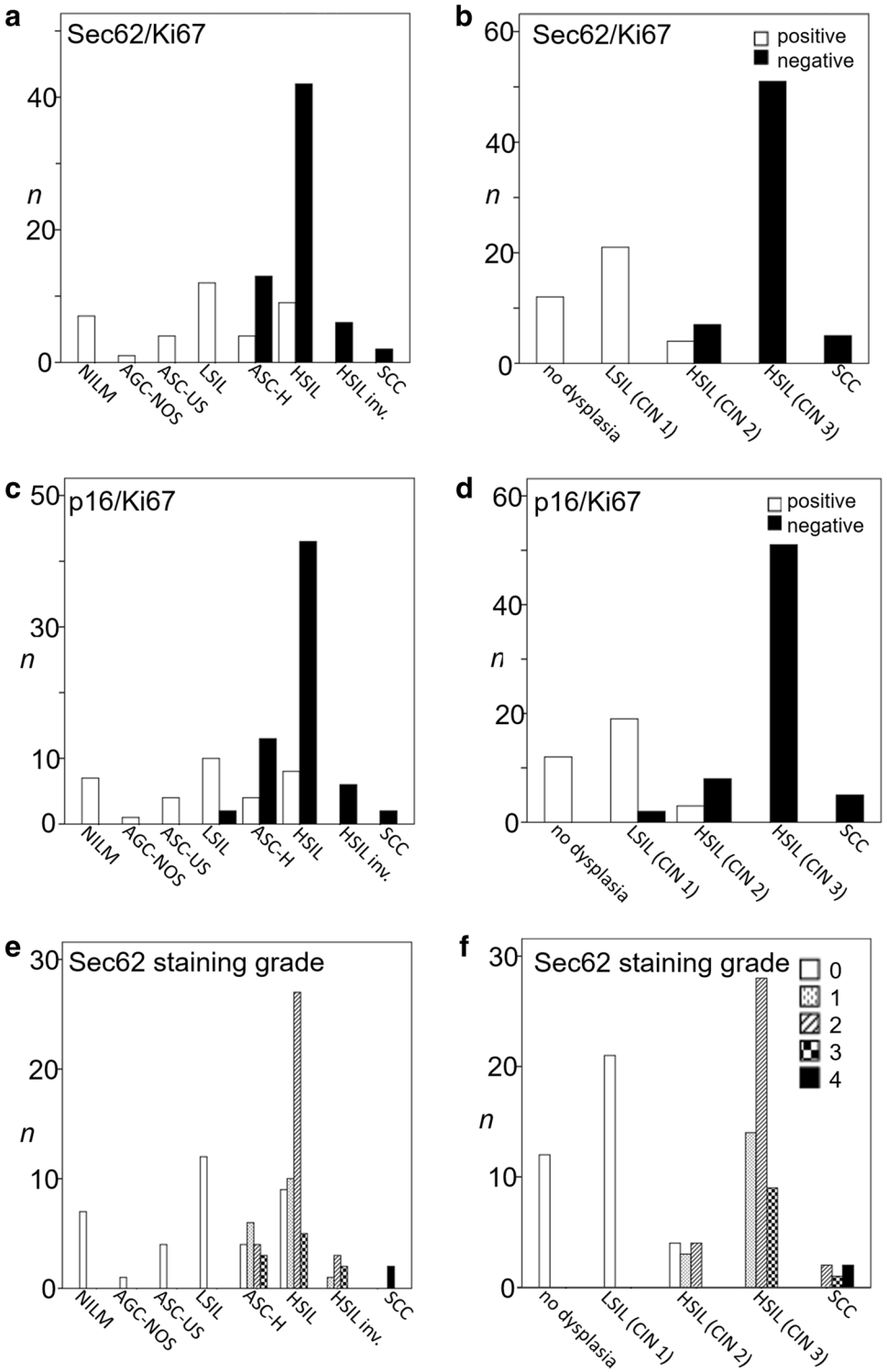

Fig. 2 Sec62/Ki67 dual staining (**a**, **b**), p16/Ki67 dual staining (**c**, **d**) and Sec62 immunostaining intensity grade (**e**, **f**) according to cytological (**a**, **c**, **e**) and histological (**b**, **d**, **f**) findings. *NILM* negative for intraepithelial lesion or malignancy, *AGC-NOS* atypical glandular cells not otherwise specified, *ASC-US* atypical squamous cells of undetermined significance, *LSIL* low-grade squamous intraepithelial lesion, *ASC-H* atypical squamous cells, cannot exclude HSIL, *HSIL* high-grade squamous intraepithelial lesion, *HSIL inv.* high-grade squamous intraepithelial lesion suspicious for invasion, *SCC* squamous cell carcinoma

such as Sec62/Ki67 dual staining could achieve, in addition to greater oncological safety, a reduction in the incidence of serious obstetrical complications. Thus, the goal of adding new methods to the established screening methods of cytology and/or HPV testing is to minimize the possibility of damage from false-positive results.

Conclusions

Sec62/Ki67 dual-staining ICC is a promising cytological tool for interpreting high-grade squamous lesions in cytological specimens and for better assessing the risk of progression to SCC. Further studies are needed to evaluate the

Table 2 Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Sec62/Ki67 and p16/Ki67 dual staining

	Sec62/Ki67 dual staining		p16/Ki67 dual staining	
	CIN2+	CIN3+	CIN2+	CIN3+
Specificity (%)	100	84.09	93.94	77.27
Sensitivity (%)	94.37	100	95.52	100
PPV (%)	100	88.89	96.97	84.85
NPV (%)	89.20	100	91.18	100

role of this new test in routine cervical cancer screening, for example, in the triage of patients with cytologies indicating atypical squamous cells of undetermined significance (ASC-US) and atypical squamous cells, cannot exclude HSIL (ASC-H).

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Author contributions FZT: project development, data collection and analysis, manuscript writing. JCR: review and editing. FB: visualization, review and editing. RMB: review and editing. IJ-B: review and editing. EFS: review and editing. BS: review and editing. ML: project development, data analysis, review and editing.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or 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.

Conflict of interest F. Z. Takacs and M. Linxweiler have a pending patent application for the Sec62/Ki67 immunocytochemical dual staining (application number: LU100824). All other authors declare no conflicts of interest.

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