Original paper

Expression of fascin in association with p16 and Ki-67 in cervical lesions: immunohistochemical study

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Annual gynecological examination with cervical cancer screening and HPV vaccination ensures the appropriate prevention of the onset and progression of cervical cancer. Currently, efforts are being made to find new diagnostic and prognostic biomarkers. Fascin, an actin-bundling protein, promotes cellular migration. Its overexpression has been observed in many types of squamous carcinomas and was usually correlated with a worse prognosis and metastasis. However, the data on fascin expression in cervical lesions are limited.

This study focuses on the quantitative evaluation of fascin expression, the immunoreaction intensity and subcellular localization of fascin expression in low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and squamous cell carcinomas (SCC). Fascin expression was also correlated with the routinely used diagnostic markers p16 and Ki-67. Biopsy specimens ($n = 67$) of LSIL, HSIL and SCC were taken from adult women in the age range 20–86 years.

Fascin expression was detected by immunohistochemical analysis and quantified using morphometric software. Analysis of variance confirmed statistically significant differences in the percentage of fascin-positive cells between the LSIL, HSIL and SCC groups. Finally, the results showed a significant positive correlation between fascin expression and p16 and Ki-67 expression.

Key words: squamous cell carcinoma, cervix, fascin, epithelial-mesenchymal transition, immunohistochemistry.

Introduction

Cervical squamous carcinoma is the fourth most frequently diagnosed cancer in women. More than 604 000 new patients were diagnosed and it was estimated that up to 341 000 deaths occurred during 2020 [1]. On the other hand, cervical cancer is one of the few neoplastic diseases with both primary and secondary prevention. Persistent HPV infection leads to the development of intraepithelial cervical neoplasia. After HPV infection other risk factors were identified, e.g., other sexually transmitted diseases, promiscuity, smoking, early sexual debut, and oral contraceptive pills [2]. Although cervical cancer has the means of prevention and has good treatability, its metastatic progression leads to a high number of cancer-related deaths [3]. Many studies [3–7] have already revealed the role of epithelial mesenchymal

transition (EMT) in cervical cancer because EMT correlates with aggressiveness of tumors and plays a key role in the metastatic process and resistance to chemotherapy [8].

One of many proteins involved in EMT is the globular 55-kDa actin-bundling protein fascin (FSCN1). It is an evolutionarily conserved protein with 4 β-trefoil domains firstly described in sea urchin coelomocytes [9]. Three fascin isoforms were identified in mammals. Fascin-1 (referred to here as fascin) is expressed in the mesenchymal and nervous tissues during development. In adulthood its expression is mainly restricted to the brain, endothelium, and testes. Fascin-2 (FSCN2) is expressed in retinal photoreceptor cells and in hair. The expression of fascin-3 (FSCN3) is specific for testes and developing spermatozoa [10–12]. Fascin crosslinks actin and microspikes. Furthermore, fascin contributes to the regulation of adhesion dynamics and cellular migration [10, 13]. The study of actin bundling protein-1 has become popular in connection with cancer in recent years. Fascin expression is absent or minimal in adult epithelial tissue, but is highly expressed in many types of carcinomas, e.g., colorectal [14], breast [15], head and neck [16], gastric [17], pancreas [18], and ovarian [19]. Up-regulation of fascin in carcinomas correlates with a higher ability of tumor cells to spread and a poor prognosis [20]. Based on this knowledge, fascin could become a promising tumor biomarker in addition to the standard biomarkers used in daily routine practice in the evaluation of cervical biopsy specimens as well. The present immunohistochemical study aimed to investigate fascin expression in cervical lesions and squamous carcinoma and reveal the possible association between fascin and $p16^{Ink4a}$ and Ki-67.

Material and methods

Sixty-seven tissue samples in the study were obtained from the Department of Pathology, St. Elizabeth Cancer Institute Hospital in Bratislava. For the study, formalin-fixed paraffin-embedded archival blocks were used. The following parameters were available for this study from the pathology reports: patient's age, histopathological category of lesions low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), squamous cell carcinoma (SCC) and the semiquantitative evaluation (+,++,+++) of $p16^{INK4a}$ and Ki-67, standardly used diagnostics.

Paraffin blocks were cut into 4 μm thick sections. The samples were deparaffinized with xylene and rehydrated afterwards using a series of descending alcohol. Antigen retrieval was performed by submerging the slides in retrieval solution (Target Retrieval Solution Low pH, Dako). To block endogenous peroxidase, the slides were immersed in a 3% H_2O_2 solution for 10 minutes. The histochemical staining method followed the manufacturer's protocol. Staining was performed with monoclonal anti-fascin antibody (Abcam, FSCN1/417, 1 : 1000 dilution). The primary antibody was visualized using the Envision TM FLEX/HRP system polymer technique (Dako) using the peroxidase chromogen DAB (3,3-diaminobenzidine). Counterstaining was done with Mayer's Hematoxylin (Dako). Negative controls were included with each stained group excluding the primary antibody. The endothelium was used as the internal positive control for the stained samples.

For the quantitative evaluation, we used computer-assisted morphometric analysis. Digital microphotographs were taken at a magnification of 400x with a camera microscope (Canon EOS 2000D) installed in an Olympus BX43 bright-field microscope. Microphotographs were analyzed with QuickPhoto-Micro Version 3.2 software (Promicra, Prague, Czech Republic) software. Each slide was examined by 2 independent observers (SHC, VM) to identify the highest expression of fascin in the lesions. At least 5 fields (hot-spot areas) from each slide were evaluated (Weidner, 1995). Expression of fascin was defined as the percentage of positively stained epithelial cells in the standard area of the lesion. The intensity of the immunoreaction was semiquantitatively assessed: 0 (none), 1 (weak), 2 (moderate) and 3 (strong). Subcellular localization of the fascin was evaluated as cytoplasmic (C) or combined cytoplasmic and nuclear (CN).

Statistical analysis

Statistical analysis was performed using JASP 0.15 software. The Kruskal-Wallis test, post-hoc Bonferroni correction, Kendall's tau b and χ^2 test were used. A p-value less than 0.05 was determined as the minimum for statistical significance.

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Results

Samples were taken from the women in the age range of 20–86 years (Table I).

As mentioned above, the samples were divided into three groups: LSIL (23), HSIL (21) and SCC (23).

The χ^2 test confirmed statistically significant differences in the expression of $p16^{Ink4a}$ ($p < 0.001$) and Ki-67 ($p < 0.001$) between the LSIL, HSIL and SCC groups. The expression of both antigens was weak in the LSIL group, higher in the HSIL group, and the strongest expression was in the SCC group.

The data of fascin expression (values from morphometric analysis of the fascin positive area) failed the parametric assumption; therefore, the Kruskal-Wallis test was used for the analysis of variance. Statistically significant differences were observed in fascin

Table I. Descriptive statistics

AGE	LSIL ($N = 23$)	HSIL ($N = 21$)	SCC ($N = 23$)
Mean	36.57	33.43	56.30
Standard	10.57	9.75	15.84
Median	39	32	54
Minimum	20	24	34
Maximum	51	69	86

HSIL – high-grade squamous intraepithelial lesions, LSIL – low-grade squamous intraepithelial lesions, SCC – squamous cell carcinomas

expression ($p = 0.044$). Post hoc Bonferroni correction revealed a significant difference between fascin expression in the LSIL group and the SCC group ($p = 0.040$). The average values of fascin positive area in the SCC group were 31% higher than the values in the LSIL group (see Table II).

The difference of fascin positive area between the LSIL and HSIL group was 11%, between the HSIL and SCC group 18%. These differences were not statistically significant.

Immunohistochemical analysis showed C or combined CN localization of fascin expression. Nuclear staining was usually observed as a diffuse pattern. Most of the C staining had a granular to diffuse pattern. The ratio of the number of samples with only C localization and combined CN localization (C/CN) of fascin was 13/10 in LSIL, 8/13 in HSIL and 13/10 in the SCC group. Statistical analysis using the χ^2 test did not reveal significant differences in subcellular localization of fascin expression ($p = 0.376$).

Frequently the intensity of immunoreaction in samples was diverse; therefore, the predominant pattern was used for scoring (weak, moderate, strong). The intensity of the reaction in LSIL tissue samples was rather moderate (12/23) and weak (6/23). The highest intensity of immunoreaction was in the basal and parabasal layer of the epithelium (Fig. 1C). A moderate (10/21) and a strong (6/21) intensity of immunoreaction was observed mainly in HSIL cases (Fig. 1D). Diffuse immunoreaction was demonstrated in the basal and parabasal layers, although more than half of the cases (13/23) showed fascin positivity in 2/3 to the full thickness of the epithelium (Fig. 1E). Squamous cell carcinoma had the strongest intensity of immunoreaction in 12/23 samples and moderate in 11/23. The combined pattern of immunoreaction was observed predominantly in cases with a strong intensity of immunoreaction of 9/12. In the combined subcellular location, we sometimes observed stronger perinuclear positivity (7/9) (Fig. 1B).

The analysis did not confirm statistically significant differences in immunoreaction intensity between the LSIL, HSIL and SCC groups ($p = 0.055$).

Statistical analysis (Kendall's tau b) confirmed a significant positive correlation between fascin and $p16^{Ink4a}$ expression ($r = 0.239$; $p = 0.012$) and fascin and Ki-67 expression ($r = 0.238$; $p = 0.010$) (see Table III).

The expected correlation was confirmed between $p16^{Ink4a}$ and Ki-67 ($r = 0.825$; $p = 0.000$), $p16^{Ink4a}$ and age ($r = 0.265$; $p = 0{,}006$), Ki-67 and age ($r = 0.352$; $p = 0.000$). However, the correlation between fascin expression and age was not statistically significant ($r = 0.066$; $p = 0.435$).

Discussion

Fascin is one of many proteins involved in the EMT process that has a key role in cancer pathology.

Table II. Statistical analysis of data using Kruskal-Wallis test, post-hoc Bonferroni correction and χ^2 test

Fascin expression	LSIL ($N = 23$)	HSIL ($N = 21$)	SCC ($N = 23$)
Fascin positive area	12.479 ±6.735	13.835 ±6.026	16.312 ±5.759
Kruskal-Wallis test		$p = 0.044$	
Post hoc Bonferroni correction		LSIL vs. HSIL $p = 1.000$ HSIL vs. SCC $p = 0.398$ LSIL vs. SCC $p = 0.040$	
Immunoreaction intensity			
Weak	6	5	0
Mild	12	10	11
Strong	5	6	12
χ^2		$p = 0.376$	
Subcellular localization			
Cytoplasmic	13	8	13
Nuclear and cytoplasmic	10	13	10
χ^2		$p = 0.055$	

HSIL – high-grade squamous intraepithelial lesions, LSIL – low-grade squamous intraepithelial lesions, SCC – squamous cell carcinomas

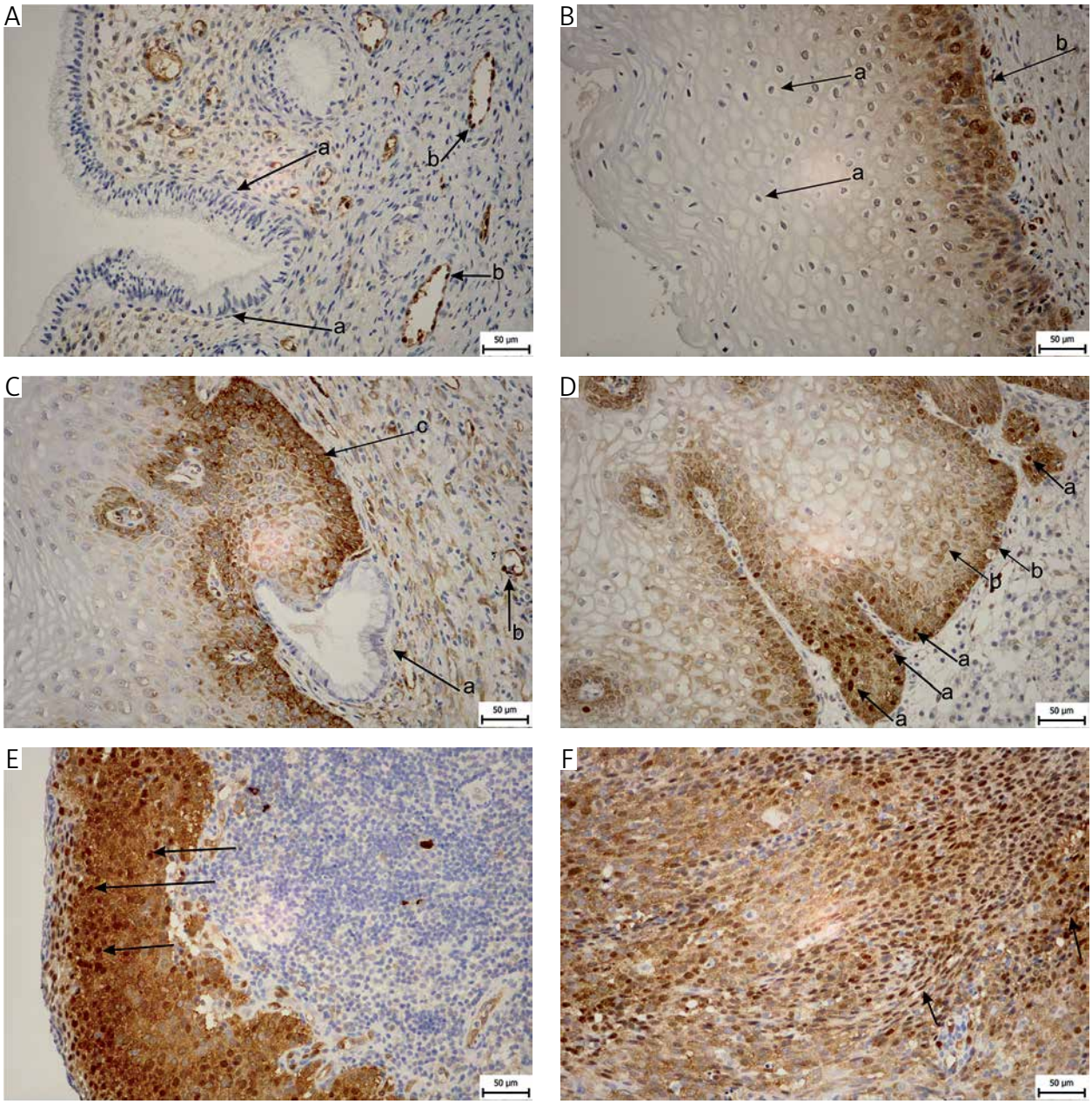

Fig. 1. Fascin expression. **A**) Endocervical epithelium negative for fascin (arrows-a) and positive endothelial cells (arrows-b); B) low-grade squamous intraepithelial lesions showing basal and parabasal (arrow-b) positivity for fascin expression with cytoplasmic and perinuclear localization, arrows-a pointing to cells with koilocytic appearance that are negative for fascin; **C**) endocervical gland epithelium negative for fascin (arrow-a), positive endothelial cells (arrow-b), and high-grade squamous intraepithelial lesions (HSIL) with moderate intensity of fascin immunoreaction localized in the basal and parabasal layer (arrow-c); **D**) fascin positive HSIL lesion with combined nuclear-cytoplasmic (arrows-a) and cytoplasmic (arrows-b) subcellular localization; **E**) squamous cell carcinomas (SCC) showing strong positivity for fascin with cytoplasmic and combined nuclear-cytoplasmic positivity (arrows); **F**) invasive SCC with both types of subcellular localization of fascin; cells are changing their shape, which is visible also on their nuclei (arrows)

Table III. Statistical analysis using non-parametric Kendall's tau b test

Correlation Kendall's tau b	P16 Ink4a expression	Ki-67 expression	Age
Fascin expression			
Kendall's tau b	$r = 0.239; p = 0.012$	$r = 0.238; p = 0.010$	$r = 0.066; p = 0.435$

It helps neoplastic cells to overcome the individual steps of the metastatic cascade [21]. This remarkable process is initialized and regulated through a wide array of factors and signaling pathways that may overlap [22]. One of the many involved proteins is an actin-bundling protein fascin, which participates in EMT through various pathways, including Wnt/β-catenin signaling [23]. As mentioned in the previous section, fascin takes part in formation of parallel bundles of actin filaments by crosslinking them through its 3 binding sites. Therefore, we find fascin involved in a variety of physiological processes, e.g., formation of cellular protrusions, promotion of cell adhesion, regulation of vesicle release, and migratory capacity [24–27]. On the other hand, fascin was identified as a supporter of neoplastic cell migration during metastasis [11]. Fascin expression is correlated with a worse prognosis and survival outcomes in many types of cancer [20].

Overexpression of fascin is typical for many different types of carcinomas even though the expression of fascin in normal adult epithelium is absent or minimal [28]. In our study we observed increasing expression of fascin through groups of LSIL, HSIL to SCC. To our knowledge, there are only 3 published studies on fascin expression in cervical lesions [29–31].

Kabukcuoglu *et al*. investigated its expression not only in intraepithelial lesions and carcinoma, but also in tissues with chronic inflammation. Their study showed basal and parabasal positivity of cells in LSIL and diffuse positivity of HSIL throughout the thickness of the epithelium. Our study showed similar results for LSIL (Fig. 1B); in the case of HSIL staining of full thickness of the epithelium was observed in only 56.52% (13/23) of the samples. Kabukcuoglu *et al*. also observed up-regulated fascin expression in malignant lesions with an increasing microvessel count and reported intravascular tumor cells intensely positive for fascin, which may support the assumption that fascin increases the migratory capacity of neoplastic cells [31].

Koay and colleagues (2014) reported similar outcomes as ours. Fascin expression in LSIL samples was localized in the basal/parabasal compartments of the epithelium and occupied two thirds to the full thickness of the epithelium in HSIL and SCC. They reported that the positivity of cells in superficially spreading SCC was located at the invasive front (tumor-host interface), which is consistent with our findings. The koilocytes were, on the other hand, fascin negative, which is consistent with our results (Fig. 1B). The epithelium of the endocervix showed no fascin expression; on the other hand, endothelial cells and dendritic cells in reactive stroma were positive, which is consistent with our findings (Fig. 1A). We made similar observations in our lesions. Koay *et al*. also reported basal and parabasal C staining in the normal mature exocervical epithelium. In contrast, we found very weak to no positivity in the normal epithelium of the exocervix.

Ghalejoogh *et al*. found an association between fascin overexpression and HPV positivity and observed that no cervical carcinoma sample in their study had stronger immunoreaction intensity than the endothelial control. In contrast, we observed the strongest intensity of immunoreaction in carcinoma samples. Their study did not report any correlation between fascin expression and age. We observed a significant negative correlation between fascin and age of the patients in the LSIL group.

The subcellular localization of the fascin was C and was observed as a diffuse to granular pattern [29–31]. In addition to the C expression of fascin, we observed combined CN localization as well (Fig. 1F).

Groen *et al*. confirmed that the nuclear and perinuclear localization of fascin is conserved from *Drosophila* to mammalian cells. Groen *et al*. described the nuclear role of fascin as a binding partner to nesprin-2, a nuclear envelope protein. This interaction is necessary for the nuclear motion, which ensures that its shape changes when passing through limited spaces of extracellular matrix. Further studies have pointed to a new role for phosphorylated fascin in the nucleus. They assume an important role in histone methylation and pol2-mediated transcription of specific target genes [32–35].

Antigens p16 ^{Ink4a} and Ki-67 became important biomarkers for cell progression and proliferation. They are nowadays used as diagnostic and prognostic biomarkers in routine bioptic procedures. p16 ^{Ink4a}, a tumor suppressor and member of the INK4a family, is a regulator of cell cycle progression. Its overexpression in HPV-mediated cervical neoplastic lesions is due to the action of oncoproteins (E6/E7) that cause degradation and inactivation of regulatory p53 and Rb proteins. This reaction leads to cell cycle progression and therefore overexpression of p16 ^{Ink4a} [36–38]. Ki-67 is a DNA replication marker with many molecular functions [39]. In this study we found a positive correlation of p16 ^{Ink4a} and Ki-67 with fascin. The correlation of fascin and Ki-67 was observed in the study of Abosarie and Ibrahim [40]. They confirmed the positive relationship between fascin expression and Ki-67 in prostatic cancer lesions. This relationship was also observed by Hashimoto *et al*. in a study of gastric carcinoma.

Conclusions

Fascin expression has been reported in many different types of carcinomas. With this study, we wanted to expand knowledge about fascin in connection with routinely used diagnostic biomarkers in practice and determine its expression in cervical lesions.

We observed an increase of fascin expression from low-grade to high-grade squamous intraepithelial lesions and SCC. Fascin expression is correlated with a worse prognosis and a higher chance of metastatic invasion due to the epithelial-mesenchymal transition process. This study showed a positive relationship between fascin and p16 ^{Ink4a} and Ki-67, which may also indicate the role of fascin in tumor progression. Based on our findings, we suppose that fascin may represent a plausible biomarker in assessment of cervical lesions.

However, further studies with a larger number of samples and more clinical-morphological information are needed to better understand fascin's function and evaluate its possible role as a biomarker in cervical lesions.

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