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ORIGINAL ARTICLE

Immunohistochemical Study EMT-Related Proteins in HPV-, and EBV-Negative Patients with Sinonasal Tumours

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Abstract Epithelial to mesenchymal transition (EMT) is a biological process in which the epithelial cells, transform to mesenchymal cells via multiple biochemical modifications. Immunohistochemical method was used to examine the expression of EMT-related proteins: Slug, E-cadherin and fibronectin, in 41 cases of sinonasal inverted papilloma (SIP), 33 cases of sinonasal squamous cell carcinoma (SNC), and 22 cases of normal mucosa as a control. In all cases negative viral status was previously confirmed using both in situ hybridization and immunohistochemical method. The immunoexpression of Slug and fibronectin were significantly increased in the SNC group as compared to SIPs and control cases. The immunoexpresssion of Slug was also higher in SIPs as compared to controls. The immunoexpression of E-cadherin was significantly lower in SNCs group as compared with SIPs and controls, but no statistically significant difference in E-cadherin immunoexpression was noted between SIPs and control cases. There were statistically significant negative correlations between immunoexpression of Slug vs E-cadherin, E-cadherin vs fibronectin and positive correlation between Slug vs fibronectin in SNC. Statistically significant correlation between Slug and fibronectin immunoexpression in SIPs was also found. In conclusion, our findings suggest that relationships between Slug, E-cadherin and fibronectin could potentially point to EMT in the sinonasal cancer. Lack of correlation between EMT-related proteins in tested SIPs could reflect a benign nature of those cases.

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Introduction

Malignant neoplasms of the sinonasal tract are uncommon, accounting for 1 % of all malignant tumors, and approximately 5 % of head and neck malignancies [1–3]. There are some rare occupational and industrial exposures (fumes, dusts from wood and leather and exposure to cadmium, nickel or chromium dusts and other rare minerals), which may account for the development of these cancers. There does not appear to be any other racial, ethnic or geographic propensity for these cancers to develop [3, 4].

Sinonasal inverted papilloma (SIP) is a one of the most common epithelial neoplasm that arises from the outlining Schneiderian respiratory membrane with origin in the lateral wall of the nose, ethmoid sinus or maxillary antrum. SIPs are particularly noteworthy because, in contrast to exophytic papillomas, SIPs have malignant behavior including recurrence tendency, destructive capability and propensity to malignancy. SIPs are concomitantly diagnosed in 1,7–56 % of patients with sinonasal squamous cancer [5–9].

Metastatic dissemination is a major cause of death in patients with cancer. It is well known that malignant transformation in many carcinomas is associated with the loss of epithelial phenotype (reduced intercellular adhesion, cytoskeletal reorganization and a loss of epithelial cell polarity) [10, 11] and gain of a mesenchymal phenotyp; a process known as epithelial-mesenchymal transition (EMT). Compared with normal epithelial cells, EMT cells display an enhanced migratory capacity, increased production of extracellular matrix components (EMC), a loss of intercellular cohesion, invasiveness and increased resistance to apoptosis. EMT cells may

detach from the basement membrane, thus EMT is a process that encourage cancer cell migration, invasion, and metastasis [11, 12]. Furthermore, EMT is also known to play a role in embryonic and organ development, wound healing and tissue remodeling [13, 14]. EMT-related proteins are expressed at the invasive fronts of primary tumors, and they can further change the composition of EMC to promote cancer cell metastasis and form metastatic tumors in remote organs through the reversed mesenchymal-epithelial transition process [11]. Various biomarkers have been used as surrogates of the EMT process, including the loss of epithelial markers - E-cadherin and cytokeratin, as well as the upregulation of mesenchymal markers such as vimentin, fibronectin, α-smooth muscle actin, and transcriptional factors - Snail and Slug [13].

Maintenance of stable cell-cell contacts and cell polarity is an essential requirement for the functionality and homeostasis of epithelial tissues in the adult organism. The E-cadherin-catenin complexes represent the main adhesion system responsible for the maintenance of cell-cell contacts in epithelial tissues [15, 16]. The intracellular domain of E-cadherin is linked to the actin cytoskeleton through critical interactions with its associated proteins catenins [15, 17]. E-cadherin functions also in cellular signal transduction and morphogenesis [18]. Several transcription factors, including Slug, Snail, Twist and zinc finger E-box-binding homeobox 1 (ZEB1), have been implicated in the transcriptional repression of E-cadherin and the induction of EMT [11, 19]. Slug (Snail2) belongs to the Snail superfamily of zinc finger transcription factors, which also includes Snail1 and Snail3 (Smuc) [20]. Snail and Slug, a related superfamily members, are expressed during development in the early mesoderm and neural crest. The superfamily is involved in cell differentiation and survival, two processes pivotal in cancer research [21–23].

Several studies published previously have highlighted the role of viral antigens in modulation of tumorgenesis [24–29]. Of the various human viruses, human papillomavirus (HPV) and Epstein-Barr virus (EBV) are the most important tumorigenic factors. Human papillomavirus is a small DNA virus showing an affinity to the stratified squamous epithelium found on the mucosa and skin. Numerous data suggest that HPVs are the main viruses etiologically involved in the development of squamous cell carcinomas [6, 30]. Between 15 % and 35 % of head and neck squamous cell cancers could be associated with high risk HPVs, in particular HPV 16 [5, 6, 31]. HPV types 6 and 11 have been the most frequently identified HPV subtypes in oral and sinonasal papillomas [7, 9, 31, 32]. Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus which is associated with the development of tumors of both lymphoid and the epithelial origin. It has been found to be associated with various lymphoid and epithelial malignancies which include Burkitt's lymphoma, Hodgkin disease and nasopharyngeal cancer [33]. Recent studies showed that viral antigens can modulate action of the same signaling pathway as EMT (tyrosine kinase receptor signaling, Hedgehog and Notch signaling, TGFß, Wnt/ß-catenin, and Smad complex) [24, 25, 34]. Unfortunately, most of the studies did not determine or report viral status.

Little is known about the expression of EMT-related proteins in sinonasal lesions. Therefore, the aim of this study was to evaluate the immunoexpression of Slug, E-cadherin and fibronectin in HPV- and EBV-negative cases of SIPs and SNC. Another purpose was to find whether the immunoexpression of Slug could correlate with the immunoexpression of E-cadherin, fibronectin and the clinical outcome.

Materials and Methods

Patients

Forty one formalin-fixed, paraffin-embedded tissue specimens of sinonasal inverted papillomas (SIP), thirty three sinonasal squamous cell carcinomas (SNC; GII grade) and twenty two control cases (normal mucosa, noncancer affected patients) were retrieved from archival material (Chair of Pathomorphology, Medical University of Lodz, Poland). Paraffin-embedded tissue sections taken from postoperative material were diagnosed using a standard haematoxylin and eosin staining and the histological diagnoses were established according to the current standards [35]. The main criteria for patients selection were histopathological similarities within group, the same anatomical localization of lesions and the negative status of HPV as well as HBV infection. The age range for SIPs was from 26 to 82 years (mean 60.8), for SNC 49 to 78 years (mean 64.2) and for control cases 20 to 61 (mean 42,4).

To find the possible relationship between the expression of EMT markers and clinical course, patients with SNC were additionally divided into two groups: with favorable clinical course (without metastases, $n = 19$), and with poorer clinical outcome (with metastases to regional lymph nodes, $n = 14$).

Immunohistochemistry

Paraffin-embedded, 3-μm tissue sections were mounted onto SuperFrost slides, deparaffinized in xylene and ethanol of graded concentrations. For antigen retrieval, the slides were treated in a microwave oven in a solution of TRS (Target Retrieval Solution, pH 6.0, Dako) for 30 min (2 × 6 minutes 360W, 2 × 5 180W, 2 × 4 minutes 90W). After cooling down at room temperature, they were transferred to 0,3% hydrogen peroxide in methanol, for 30 min, to block endogenous peroxidase activities. Sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark) and incubated 30 min with monoclonal mouse primary antibodies against: E-cadherin (Dako; clone: NCH-38, dilution 1:50), HPV (Dako; clone: K1H8,

dilution 1:50, EBV (Dako; clone: CS.1–4, dilution 1:25), Slug (Santa Cruz, clone: A-7; dilution: 1:500, overnight at 4°C), and with rabbit polyclonal antibody against fibronectin (Dako; dilution: 1:500). Immunoreactive proteins were visualized using adequate EnVision-HRP kit (Dako, Carpinteria, CA, USA) according to the instructions of the manufacturer. Visualisation was performed by incubation the sections in a solution of 3,3′-diaminobenzidine (Dako, Denmark). After washing, the sections were counterstained with Mayer's hematoxylin and mounted. For each antibody and for each sample a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

In each specimen staining intensity of E-cadherin and fibronectin were recorded semiquantitatively by two independent observers in 7–10 adjacent high power fields and graded from 0 (staining not detectable), 1 (weak immunostaining), 2 (moderate immunostaining intensity) and 3 (strong staining). The mean grade was calculated by averaging grades assigned by the two authors and approximating the arithmetical mean to the nearest unity.

In Situ Hybridisation

All tested sections have been analyzed using commercially available probes: for HPV, DNA probe (Y1443, Dako, Carpinteria, California, USA), and for EBV, PNA probe with fluorescein (Y5200, Dako, Carpinteria, California, USA). Initially, a wide spectrum biotinylated probes for common HPV subtypes was used, according to the manufacturer's suggested protocol. The wide spectrum probe targets the genomic DNA of HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51 and 52. Further subtyping was carried out at the same way, using specific probes for HPV high risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). Afterwards, secondary antibody against fluorescein (Polyclonal Rabbit Anti-FITC/HRP Rabbit F(ab'), P5100, Dako, Carpinteria, California, USA) for EBV and catalyzed signal amplification system prepared according to the instructions of the manufacturer for HPV were used (GenPoint, CSA System for in situ hybridization; Dako, Carpinteria, California, USA). Visualisation of EBV was performed by incubation the sections in a solution of 3, 3′-diaminobenzidine (Dako, Denmark). After washing, all sections were counterstained with Mayer's hematoxylin and mounted.

Morphometry

Slug was evaluated using computer image analysis system consisting of a PC computer equipped with a Pentagram graphic tablet, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and color TV camera Panasonic (Japan) coupled with Carl Zeiss microscope (Germany). This system was programmed (MultiScan 18.03 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function).

The coloured microscopic images were saved serially in the memory of a computer, and then quantitative examinations had been carried out. The percentage of nuclei expressing Slug antigen was estimated by counting 100 cells in ten monitor fields (0,0205 mm^2 each), marking immunopositive cells, so that in each case 1000 cells were analyzed.

Results

Slug immunoexpression was strongly detected in the nuclei, immunoexpression of E-cadherin was detected in the membrane and fibronectin protein was expressed in extracellular space and matrix.

The morphometric data of immunoexpression of Slug, and the results of a semiquantitative evaluation of E-cadherin and fibronectin in SIP, SNC and control group are given in Table 1. The proportion of cells with immunoexpression of Slug was significantly increased in the SNC group (Fig.1) as compared to SIPs and control cases. The immunoexpresssion of Slug was also higher in SIPs group (Fig. 2) as compared to control group. The immunoexpression of E-cadherin was significantly lower in SNC group (Fig. 3) as compared with SIPs (Fig. 4) and control group, but no statistically significant difference in E-cadherin immunoexpression was noted between SIPs and control cases. The fibronectin immunoreactivity in the SNC group (Fig. 5) was significantly increased as compared to both SIPs (Fig. 6) and control cases. No statistically significant difference in fibronectin immunoexpression was found between SIPs and control cases.

In SNC group patients with favourable clinical course, the immunoexpression of Slug was significantly lower than in the group with poorer clinical outcome, whereas the immunoexpression of E-cadherin and fibronectin did not differ significantly in both tested group of patients with SNC. (Table 2).

The correlations between the immunoexpression of Slug, E-cadherin and fibronectin in patients with SNC, SIP and controls are presented in Table 3. In SNC there was statistically significant negative correlation between immunoexpression of Slug and E-cadherin, whereas in SIP and control cases this correlation was also negative but this was not statistically significant.

The immunoexpression of Slug was significantly correlated with the immunoexpression of fibronectin in SIP and SNC group. No statistically significant correlation between these parameters in control cases was found.

In SNC groups there were statistically significant negative correlations between immunoexpression of E-cadherin and fibronectin. No statistically significant correlations were found between these parameters in SIP and control cases.

Table 1 The immunoexpression of Slug, E-cadherin and fibronectin in sinonasal cancers (SNC), inverted papillomas (SIP) as well as in controls

Number of cases	Slug (%)	E-Cadherin (mean score)	Fibronectin (mean score)
SNC (n = 33)	46.2 ± 21.42	0.71 ± 0.58	2.32 ± 1.28
SIP (n = 41)	31.1 ± 11.8	1.12 ± 0.82	1.81 ± 0.68
Controls (n = 21)	11.1 ± 8.81	1.41 ± 0.48	1.44 ± 0.91
P value	<0.001* <0.001**	<0.02* <0.001**	<0.04* <0.008**
	<0.001***	=0.12(NS)***	=0.056(NS)***

Data are expressed as mean ± standard deviation

NS-not significant

*Between SNC and SIP

** Between SNC and controls

*** Between SIP and controls

The results of in situ hybridization as well as immunohistochemical staining indicated that HPV and EBV is not present in all tested groups.

Discussion

Several studies have revealed that EMT markers are closely related to clinicopathological features in various epithelial cancers. The clinical importance of Slug expression is well known for poor prognosis in various carcinomas, including ovarian, urothelial, hepatocellular carcinomas, breast cancer, and non-small cell lung carcinomas [22, 36–40]. Little is known about the biologic and prognostic significance of the EMT-related proteins expression in sinonasal lesions. To our knowledge studies concerning immunoexpression of EMT markers in sinonasal region are notably scanty.

EMT is a complicated process through which epithelial cancer cells acquire a reversible change in phenotype. The key step of this process is the loss of E-cadherin expression. Our study showed significantly decreased E-cadherin immunoexpression in SNC group as compared with SIPs and control group. The loss of E-cadherin expression or functional perturbations of the E-cadherin-catenin complexes have been found to occur very frequently during the progression of carcinomas [41]. It is well established that the loss of E-cadherin expression can lead to loss of contact inhibition, unlimited proliferation, dedifferentiation, and loose intercellular connections which enhance invasive and migratory features of cancer cells [42–44]. Previous studies indicated that downregulation of E-cadherin during the EMT was regulated by the activation of transcriptional repressors such as Snail, Slug, and Sip1 [45], Twist, ZEB1, and ZEB2 [46, 47]. Our study showed significantly increased immunoexpression of Slug in SNC group as compared to SIPs and the controls. Similarly to our results, Gasparotto et al. [48], also observed increased Slug expression in head and neck squamous cell carcinomas compared to normal mucosa. Numerous experiments have demonstrated that there is an inverse correlation between E-cadherin expression and Slug expression in human cancers [17, 19–23]. In our study, we have also observed statistically significant negative correlation between immunoexpression of Slug and E-

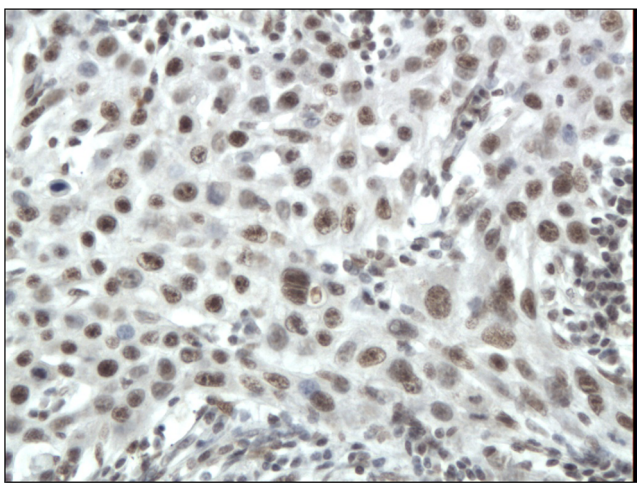

Fig. 1 Nuclear immunoexpression of Slug in sinonasal squamous cell carcinoma (SNC). Total magnification × 200

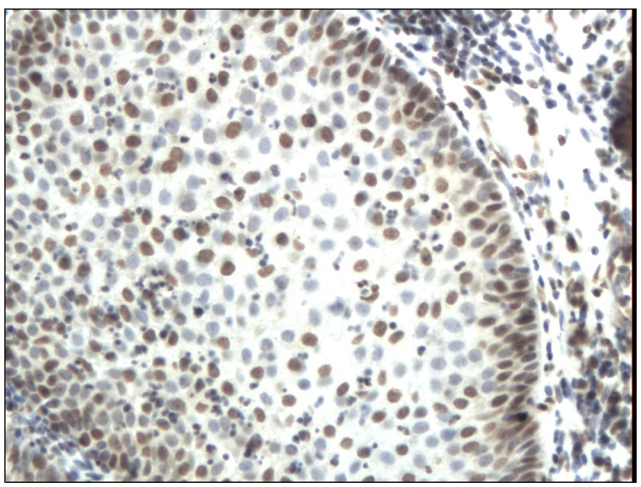

Fig. 2 Nuclear immunoexpression of Slug in sinonasal inverted papilloma (SIP). Immunohistochemistry. Total magnification × 200

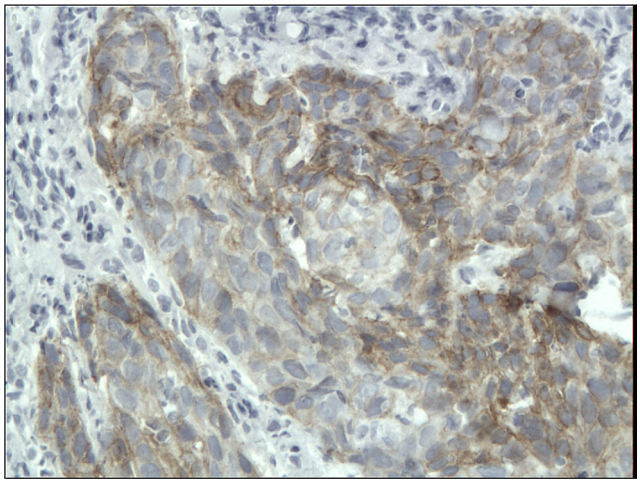

Fig. 3 Membrane immunoexpression of E-cadherin in sinonasal squamous cell carcinoma (SNC). Total magnification × 200

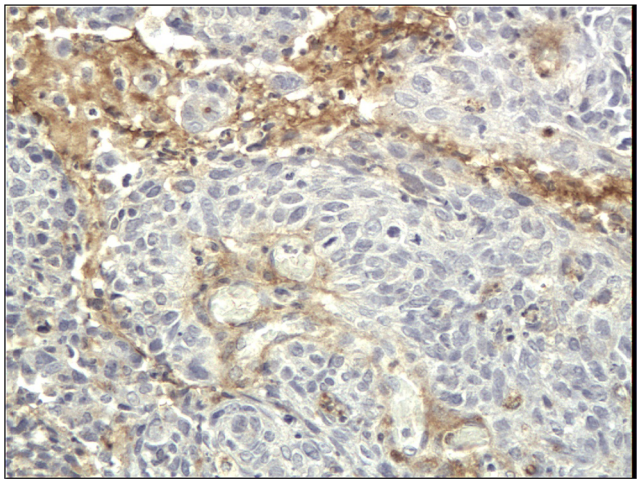

Fig. 5 Immunoexpression of fibronectin in sinonasal squamous cell carcinoma (SNC). Total magnification × 200

cadherin in SNC. Similarly to our results, Hajra et al. [49] demonstrated that Slug expression was negatively correlated with E-cadherin expression in breast cancer. In human bladder cancer, Yu et al. [50] also showed that Slug expression was correlated with E-cadherin downregulation, but in animal model of bladder cancer, Slug overexpression did not affect E-cadherin expression [51]. Other investigators also reported differential relationship between Slug and E-cadherin. Hotz et al. [52] observed that although Slug was expressed in 78% of pancreatic cancer cells, 52% of those maintained high E-cadherin expression level, and E-cadherin expression was not correlated with clinical parameters. We speculate, that differences concerning relationship between Slug and E-cadherin described above, may indicate that mechanisms responsible for the regulation and action of EMT, involve several major and minor factors, employing different signalling pathways. Moreover, literature data revealed that early and late stage EMT have different molecular profiles, suggesting that the utilization of EMT markers may be insufficient to capture the dynamic EMT process [53]. Despite the extensive research reported on signalling networks responsible for EMT, much remains to be understood regarding this complex and dynamic cellular process.

Therefore, we demonstrated that the expression level of E-cadherin was significantly downregulated, while Slug and fibronectin were upregulated in SNC compared with SIPs and controls. Moreover, some tested EMT-related markers showed significant pairwise correlation in SNC. There were statistically significant negative correlations between immunoexpression of Slug and E-cadherin, E-cadherin and fibronectin and positive correlation between Slug and fibronectin. All these findings indicated a possible occurrence of EMT in tested SNC and are in concordance with previous studies [45].

In the present study, only Slug immunoexpression was significantly increased in group of patients with unfavorable clinical course as compared to SNC group with better clinical outcome, whereas the immunoexpression of E-cadherin and

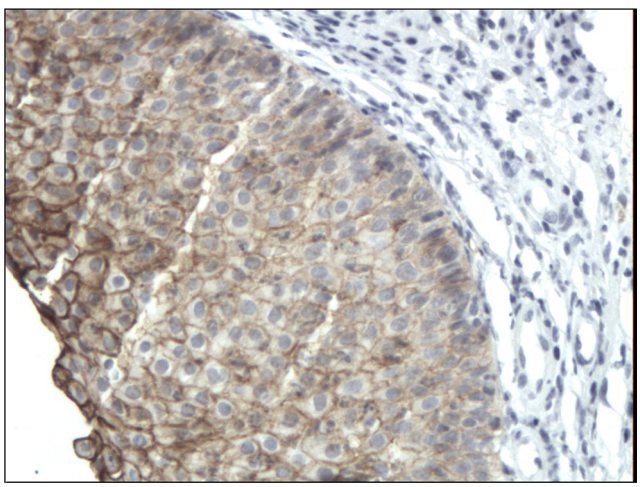

Fig. 4 Membrane immunoexpression of E-cadherin in sinonasal inverted papilloma (SIP). Total magnification × 200

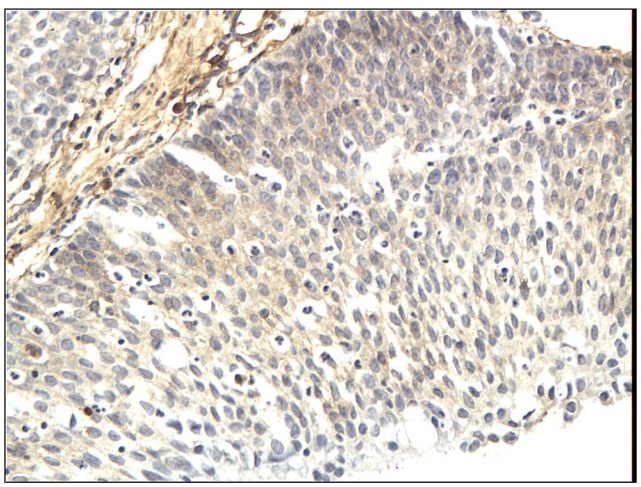

Fig. 6 Immunoexpression of fibronectin in sinonasal inverted papilloma (SIP). Total magnification × 200

Table 2 The immunoexpression of Slug, E-cadherin and fibronectin in sinonasal cancers (SNC) with favorable clinical course and in group with poorer clinical outcome

SNC	Slug (%)	E-cadherin (mean score)	Fibronectin (mean score)
Patients with favourable clinical course (n = 19)	35.2 ± 14.52	0.87 ± 0.48	2.22 ± 1.12
Patients with poorer clinical course (n = 14)	50.1 ± 23.78	0.72 ± 0.42	2.51 ± 1.48
P value	<0.04	=0.35(NS)	=0.52(NS)

Data are expressed as mean ± standard deviation. NS-not significant

fibronectin did not differ significantly in both tested group of patients with SNC. According to literature data, low E-cadherin expression and high expression of mesenchymal markers are associated with an increase in metastasis rate and poor patients prognosis [10, 13, 16], whereas Slug is well characterized as the most influential factors in E-cadherin expression and EMT process [22, 23]. The limitations of the study include a relatively low number of studied cases. We should also take into consideration that EMT process represent just one strategy of cancer invasion and metastasis. EMT process is intimately linked to malignant behavior and understanding EMT biology will be essential to improve patient outcome.

To the best of our knowledge, this is the first study that shows immunoexpression of EMT-related proteins in SIPs. In the present study, only Slug immunoexpression was significantly increased in SIPs in comparison with the control cases. Elevated immunoexpression of Slug was accompanied by slightly decreased E-cadherin immunoexpression, but there was no statistically significant correlation between them. Koo et al. [54] described significantly downregulated immunoexpression of E-cadherin in SIPs. Moreover, these authors believed that decreased immunoexpression of E-cadherin may have prognostic significance and may help predict malignant transformation of SIP into SNC. Furthermore, we found statistically significant correlation between Slug and fibronectin in SIPs but immunoexpression of fibronectin in SIPs was on the similar level to that seen in control cases. Possible explanation can be small number of tested cases, but it is also possible that technical reasons may be responsible for our results. On the other hand, all tested cases of SIPs were histologically benign neoplasm (without dysplasia), whereas EMT process constitutes an important mechanism in the development of tumor invasiveness. Thus, lack of the EMT-related correlation could confirm a benign nature of tested cases. According to Lu et al. [55] malignant behavior of SIPs (local aggressiveness, recurrence and malignant transformation) are related to HPV infection, and the higher infection rate of high risk HPV subtype is one of the reasons for malignant transformation. In our study, negative HPV status of SIPs could support this hypothesis. Histological features of tested SIPs, lack of the relationship between the EMT-related proteins and negative status of HPV are consistent and could reflect a benign nature of tested SIPs. Even so, we postulate that further studies are needed to determine the possible role of EMT in malignant transformation of SIP into SNC.

Several studies published previously have highlighted the role of viral antigens in modulation of tumorigenesis [24–29]. Gaur et al. [24] observed that presence of EBNA1 or EBNA3C result in upregulation of transcriptional repressor - Slug and Snail, a mesenchymal marker - vimentin and downregulation of a cell adhesion molecule - E-cadherin. Horikawa et al. [25] have shown that LMP contribute to EBV-associated malignancies by upregulating group of metastasis-related factors. Importantly, LMP1 has been shown to induce epithelial to mesenchymal transition by inducing transcription factors Snail and Twist in nasophryngeal carcinoma [26]. In nude mice model, Kaul et al. [27] reported that MDA-MB-231T cells expressing EBV latent viral antigens EBNA3C and/or EBNA1 had a propensity for increased metastases to the lung. The role of HPVs in the pathogenesis of head and neck squamous cell carcinomas is uncertain, mainly because detection of HPV DNA is highly variable. Only 15 % to 35 % of head and neck squamous cell cancers could be associated with HPV infection [32, 55]. It is well known that the etiology of head and neck cancers is complex and the HPV infection is related to the pathogenesis of sinonasal lesions, but not as a major factor [28]. As we postulated previously, HPV infection play a synergistic role in the multifactorial etiology of these lesions [29]. Many important signalling pathways can activate EMT in normal and cancer cells (tyrosine kinase receptor signaling, Hedgehog and Notch signaling, TGFß, Wnt/ß-catenin, and

Table 3 The relationships between the immunoexpression of Slug, E-cadherin as well as fibronectin in sinonasal cancers (SNC), inverted papillomas (SIP) and controls

Pair of variables	SNC (n = 33)	SIP (n = 41)	Controls (n = 21)
Slug vs e-cadherin	$r = -0.46$, $p < 0.008$	$r = -0.30$, $p = 0.056$(NS)	$r = -0.21$, $p = 0.36$(NS)
Slug vs fibronectin	$r = 0.38$, $p < 0.3$	$r = 0.31$ $p < 0.05$	$r = 0.17$ $p = 0.46$ (NS)
E-cadherin vs fibronectin	$r = -0.51$, $p < 0.003$	$r = -0.27$ $p = 0.08$ (NS)	$r = -0.19$ $p = 0.41$ (NS)

NS-not significant

Smad complex) [10]. On the other hand, recent studies have shown that viral antigens can modulate action of those pathways. Unfortunately, most of the studies did not determine or report viral status. Differences concerning immunoexpression of EMT markers in human cancers described in previous studies [49–52, 55], may indicate that mechanisms responsible for the regulation of EMT in various tumors are different and not fully explored. In this context, HPV- and EBV-negative cases of SIPs and SNCs could provide an insight into multiple signaling pathways of EMT without viral modulation.

In conclusion, our findings may suggest that immunoexpression and relationship between Slug, E-cadherin and fibronectin could potentially point to EMT in the sinonasal cancer. Profound analysis of molecular mechanism of actions may clarify the function of EMT in invasion and migration of cancer cells in this region. The data concerning of the role of EBV and HPV infection in EMT process in SIP and SNC still need further confirmation.

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