Pathology - Research and Practice 215 (2019) 144–150

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Immunohistochemical analysis of vocal cord polyps applying markers of squamous cell carcinogenesis

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ARTICLE INFO

Keywords:
Carcinogenesis
Hyperplasia
Immunohistochemistry
Laryngeal polyps
Molecular markers

ABSTRACT

Objective: The aim of the study was to perform a pathohistological and immunohistochemical analysis of squamous cell (SC) carcinogenesis markers on epithelial linings of vocal cord polyps.

The vocal box, being a heavily burdened organ with intensive cell renewal and regenerative processes, is therefore a favourable environment for constant epithelial growth and hyperplasia. In our ongoing projects on laryngeal carcinogenesis and research on laryngeal tissue, we encountered atypia on diagnosed nodules and polyps that are usually considered as benign formations, resulting from the above-mentioned cell renewal and regeneration, which lead to further investigation.

The purpose was to see if changes in molecular markers of SC carcinogenesis follow, or, may appear in immunohistochemical (IHC) analysis, before histological atypia in standard haematoxylin-eosin (HE) staining, and contribute in early diagnosis of potentially suspect polyps.

Methods: After classical pathohistological (PH) analysis on HE slides, IHC analysis of EGFR, cyclin D1, p53, Ki-67, and IMP3 was performed on tissue microarrays of laryngeal tissue (50 samples), ranging from normal to hyperplastic lesions with no atypia (34 samples), low-grade atypia (11 samples), and high-grade atypia (5 samples).

Results: This study established an increase and correlation of EGFR, cyclin D1, p53, Ki-67 and IMP3 IHC expressions with pathohistological findings of dysplasia in glottic polypoid lesions. Low and high-grade dysplasia had statistically higher percentages of EGFR-positive cells than normal epithelium and simple hyperplasia (SH) (low vs. normal/SH $P = 0.007$; high vs. normal/SH $P = 0.001$). High-grade dysplasia had statistically more positive cells than low-grade dysplasia ($P = 0.004$), and low-grade dysplasia had statistically more positive cells than specimens without atypia ($P = 0.007$). The percentage of positive cells was statistically higher for cyclin D1, p53 and Ki-67 in high-grade dysplasia versus low-grade dysplasia (cyclin D1 $P = 0.011$, p53 $P = 0.002$; Ki-67 $P = 0.026$; respectively) and versus normal epithelium and SH (cyclin D1 $P = 0.003$; p53 $P = 0.001$; Ki-67 $P = 0.002$; respectively). An increase of IMP3-positive cells with an increase of atypical changes in the laryngeal epithelium, from superficial towards basal layers was noticed, contrary to the usually seen positivity pattern of SC carcinogenesis markers from basal to superficial layers. A statistically significant difference of IMP3 IHC staining between the pathohistological groups ($P = 0.003$) was recorded.

Conclusion: Only polyps that present with simple hyperplasia as the greatest mucosal change can be considered as benign formations.

Pathohistologically detected atypia in polypoid changes of vocal cords, confirmed by molecular atypia with an increase of SC carcinogenesis markers, suggest their inclusion in studies of laryngeal carcinogenesis.

Our results suggest that in problematic cases IHC analysis could be of interest in detection of biological aggressiveness in polypoid laryngeal tissue and beneficiary for polyp patients' follow-up.

Further research of laryngeal carcinogenesis markers and their meaning in fibrovascular polyps is of interest.

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https://doi.org/10.1016/j.prp.2018.11.001
Received 28 September 2018; Accepted 6 November 2018
0344-0338/

1. Introduction

One of the most common symptoms that ENT patients present with is hoarseness. How to treat a patient with hoarseness still remains a subject of many debates with interdisciplinary disagreement among otorhinolaryngologists, voice specialists, and pathologists [1], especially when it comes to polypoidly-presented changes of vocal folds that cannot be lightly considered as always benign. A good pathohistological analysis in accordance with clinical finding is the key to revealing the precise diagnosis of a polypoid lesion and to successful and adequate treatment.

However, polyps and nodules are usually considered as benign growths that result from regenerative mechanisms after vocal abuse, trauma, and haemorrhage [2,3].

In this research we did not discuss, as previously done by many authors, the mechanisms of formation of vocal polyps and nodules nor investigate regenerative markers such as collagens, fibronectin etc. [2–4] The purpose of *this* research was to search for atypia in clinically diagnosed polypoid tissue usually considered as benign, and to analyse its correlation with molecular atypia. The burning question was if pathohistologically detected atypia was backed up, and to what extent, with an increase of SC carcinogenesis markers. The other question that arose was, what the meaning of these markers in polypoid lesions is.

In the previous project [5], as well as in the following research on laryngeal hyperplastic and cancerous lesions [6], we detected atypia in biopsy samples retrieved from microlaryngoscopy (MLS) specimens of patients diagnosed as polyps. This was the basis and incitement for further investigation on the subject. This research focused on epithelial IHC analysis which gave new insight into markers usually analysed in studies of laryngeal carcinogenesis that had not previously been researched on such tissue. The event prompted the investigation with EGFR and commonly used markers of laryngeal carcinogenesis, such as p53, Ki-67 and cyclin D1 [7–9] in hyperplastic polypoid lesions in order to confirm or question the adequacy of vocal polyps and nodules as control groups in studies of laryngeal carcinogenesis. Furthermore, IHC of insulin-like growth factor mRNA-binding protein 3 (IMP3) as a novel marker recently much investigated [10–12] in studies of tumorigenesis provided the authors with novel information which were not found in the literature previously.

IMP3 is an oncofetal protein involved in embryogenesis, which expression is associated with a number of malignant neoplasms, aggressive and advanced cancers. It promotes tumor cell proliferation, adhesion and invasion, and is specifically expressed in malignant tumors but not found in adjacent benign tissues [12].

Still, there is only one published research about IMP3 on laryngeal tissue [10] and only a few available data on this oncofetal protein in head and neck oncology [11,13]. These we the reasons for its inclusion in this research.

2. Methods

The research was performed on biopsy samples of polypoid laryngeal tissue obtained from the Department of Pathology and Otorhinolaryngology Head and Neck Clinic of the Clinical Hospital Center Rijeka as part of the project “An EGFR molecular marker in stage evaluation and therapy protocol of glottic carcinoma”. The Ethics Committee of Clinical Hospital Center Rijeka gave approval for the study, and consent forms were signed by the patients before enrollment.

2.1. Pathohistological (PH) and immunohistochemical analysis (IHC)

Under light microscopy, the pathohistological analysis of surgical specimens was performed, with the main focus on epithelial changes in polypoid samples presenting with some grade of atypia. Classic pathohistological findings were graded according to the latest classification [14].

IHC analysis of four molecular markers commonly used in laryngeal carcinogenesis (EGFR, p53, cyclin D1, and Ki-67), as well as a novel marker IMP3 was than performed on 50 random biopsy samples, ranging from normal to hyperplastic lesions with no atypia (34 samples), low-grade atypia (11 samples), and high-grade atypia (5 samples).

TMA technology was used to perform a uniform analysis of protein expressions of different markers on the same study material.

2.2. Tissue microarray (TMA) construction

HE-stained sections of glottic mucosa were used to mark areas with surface epithelial layers, avoiding areas of necrosis. Three tissue cores, each 1 mm in diameter, were placed in a recipient paraffin block using a manual tissue arrayer (Alphelys, Plaisir, France). Normal liver tissue was used for slide orientation. Cores were spaced at intervals of 0.5 mm in the x- and y-axes. One section from each TMA block was stained with haematoxylin-eosin for morphological assessment. Serial sections were cut from TMA blocks for IHC staining. Five μm thick sections were placed on adhesive glass slides (Capillary Gap Microscope Slides, 75 μm, Code S2024, DakoCytomation, Glostrup, Denmark), left to dry overnight at 37 °C and stored in the dark at + 4 °C.

2.3. Immunohistochemical (IHC) analysis

Paraffin-embedded, 4 μm thick tissue sections were put on silane coated microscope slides and dried overnight at 37 °C. Tissue sections were then deparaffinized in xylene three times during 10 min and rehydrated by washing in absolute and diluted ethyl alcohol (100%, 96% and 70% ethyl alcohol, 5 min each) and distilled water (10 min). For antigen epitope retrieval heat induced pretreatment was used.

For Ki-67 detection, slides with tissue sections were first submerged in Tris/EDTA pH 9 buffer (10 mM of Tris base and 1 mM of EDTA solution) and then placed in a water bath during 15 min, thermostated at 95–100°C. For IMP3, p53 and cyclin D1 detection, slides with tissue sections were submerged in Target Retrieval Solution using PT instrument for 20 min at 97°C. For EGFR and nEGFR detection, slides with tissue sections were submerged in citrate pH 6 buffer and then placed in Pascal Laboratory Pressure Cooker (Dako Cytomation, Glostrup, Denmark) for 30 s at 125°C. After cooling down for 20 min and rinsing with distilled water, the slides were ready for immunohistochemical staining.

The following antibodies and dilutions were used: Ki-67 mouse monoclonal antibody IgG1, Clone MIB-1 (1:100, Cytomation, Glostrup, Denmark), 30 min incubation; EGFR mouse monoclonal antibody, Clone EGFR.25 (1:50, Leica Biosystems Newcastle Ltd, Newcastle, UK), 60 min incubation; Cyclin D1 rabbit monoclonal antibody, Clone Ep 12 (1:50, Dako Cytomation, Glostrup, Denmark), 60 min incubation; p53 mouse monoclonal antibody, Clone DO-7 (ready-to-use antibody, Dako Cytomation, Glostrup, Denmark), 30 min incubation; IMP3 mouse monoclonal antibody, Clone 69.1 (1:100, Dako Cytomation, Glostrup, Denmark), 30 min incubation.

“Envision” – immunohistochemical method with REAL ENVISION DETECTION system on automatic immunostainer Autostainer plus (Dako Cytomation, Glostrup, Denmark) was used for protein expression detection following the manufacturer’s instructions.

2.4. Evaluation of immunohistochemical staining

The analysis of markers was performed and cells analysed by a method adjusted to multilayer squamous epithelium, where changes occur in different layers. The basal zone was defined as cells with stromal contact, the superficial zone represented surface cells with applanated nuclei, while the suprabasal zone was defined as the transforming layer between them.

For each immunohistochemical staining, five representative high-power visual fields (x400 magnification) of tissue samples were

examined under the light microscope (Olympus CHT, Optical. Co. Ltd.) and the percentages of positive epithelial cells were obtained. The mean value of positive cells was calculated and, upon that, integrated percentages of positivity for each marker were obtained in each polypoid tissue specimen. This mean value was used for statistical analysis of EGFR, cyclin D1, p53, and Ki-67. For IMP3 being a new marker, the analysis for purpose of comparison with the only available literature on the subject in laryngeal tissue [10], categories of intensity were used: as 1 for less than 25% of positive epithelial cells, 2 for 25–50% of positive epithelial cells, and 3 for over 50% of positive epithelial cells. Cell counting was performed by two experienced pathologists in the field of head and neck pathology, and cellular positivity based upon subjective evaluation of clear brown decoration of cellular elements specific to the antibody. EGFR staining patterns were designated as membranous, IMP3 as cytoplasmic, whereas p53, Ki-67 and cyclin D1 staining patterns were observed as nuclear stainings of epithelial cells.

Sections of placental tissue with known positivity of IMP3 were used as positive controls for IMP3 staining. Colonic cancer tissue sections over-expressing EGFR, breast cancer tissue for cyclin D1, and tonsillar tissue for p53 and Ki-67, were also used as positive controls.

3. Statistical Analysis

Statistical analysis of data was performed using Statistica for Windows, release 13.1 (StatSoft, Inc., Tulsa, OK). Normality of distribution of data was calculated by Kolmogorov-Smirinovljev test. Data of markers: EGFR, cyclin D1, p53, and Ki-67 were normally distributed and were presented as the mean ± standard deviation. One-way ANOVA was used to compare the differences among these markers between normal epithelium and SH, low- and high-grade dysplasia. The analysis of the number of cases regarding alcohol and smoking, as well as IMP3, was performed using Pearson χ^2-test.

All statistical values were considered significant at the *P*-level of < 0.05.

4. Results

4.1. Clinicopathological data

The clinicopathological data are displayed in Table 1.

One-way ANOVA showed a significant difference between different grades of atypia according to age ($F = 3.96$, $P = 0.026$).

Chi-square test did not show a significant difference between different grades of atypia according to gender ($\chi^2 = 2.23$; $P = 0.328$).

The post-hoc analysis showed that patients with high-grade dysplasia were statistically older than the other two groups (high vs. normal/SH $P = 0.037$; high vs. low $P = 0.024$).

A positive history of smoking was reported in 66% of patients and drinking in 27.7%. History of professional use of voice (teachers and singers) was recorded in 22% of patients, while 28% of patients had a positive medical history of gastro-oesophageal reflux (GORD).

Chi-square test was statistically significant between pathohistological groups for smoking ($P = 0.045$) and GORD ($P = 0.023$).

Smoking was recorded especially in low-grade dysplasia, in which 10 out of 11 patients had a positive history of smoking, while GORD was statistically higher in high-grade dysplastic lesions in which 4 out of 5 patients had a positive medical history of GORD disorders.

Professional usage of voice (teachers and singers) and alcohol did not prove to be statistically significant between the analysed PH groups. ($P > 0.05$).

Although smokers had slightly higher values of p53-positive cells (12.87% in non-smoking group vs. 13.65% positive cells among smokers, $P = 0.691$), the increase was not statistically significant.

Table 1
Demographic and clinicopathohistological data.

Pathohistological classification	n	Age (years) Mean ± SD	Gender (%)	
			Female	Male
Normal epithelium / SH	34	46.5 ± 12.3	6 (12)	28 (56)
Low-grade dysplasia	11	43.3 ± 14.8	1 (2)	10 (20)
High-grade dysplasia	5	62 ± 10.2	2 (4)	3 (6)
Total	50	47.3 ± 13.3	9 (18)	41 (82)

SD – standard deviation; SH – simple hyperplasia.

4.2. Immunohistochemical (IHC) analysis

The percentages of positive markers in different pathohistological groups are presented in Table 2.

All markers showed statistically significant differences according to different levels of atypia.

Low-grade dysplasia, as well as high-grade dysplasia, had statistically higher percentages of EGFR-positive cells than normal epithelium and SH (low vs. normal/SH $P = 0.007$; high vs. normal/SH $P = 0.001$). Also, high-grade dysplasia had statistically more positive cells than low-grade dysplasia ($P = 0.004$).

The results are graphically presented in Fig. 1.

Cyclin D1, p53, and Ki-67 had approximately the same percentages of positive cells in simple hyperplasia and low-grade dysplasia, with a slight increase in the dysplastic group, without statistical significance (cyclin D1 $P = 0.999$; p53 $P = 0.992$; Ki-67 $P = 0.685$). The percentage of positive cells was statistically significantly higher for all three markers in high-grade dysplasia versus low-grade dysplasia (cyclin D1 $P = 0.011$; p53 $P = 0.002$; Ki-67 $P = 0.026$ respectively) and specimens without atypia (cyclin D1 $P = 0.003$, p53 $P = 0.001$; Ki-67 $P = 0.002$ respectively) as shown in Table 2.

p53 and Ki-67 had very similar percentages of positive cells in all analysed groups of atypia, while cyclin D1 displayed a slightly higher percentage of cell positivity as shown in Table 2.

4.3. Staining patterns of EGFR, p53, Ki-67, cyclin D1 and IMP3 in vocal cord mucosa

Vocal cord mucosa diagnosed as simple hyperplasia revealed positive membranous EGFR reaction in the basal and, occasionally, suprabasal cells of the squamous epithelial lining, while the cells of the superficial layer were negative for EGFR (Fig. 2E).

p53, cyclin D1, and Ki-67 immunostaining showed a clear nuclear reaction in some suprabasal and sporadically basal cells which is in accordance with simple hyperplasia without atypia (Fig. 2B–D).

Low-grade dysplasia had more positive cells in suprabasal and basal layers, but positivity usually did not surpass half of the epithelial thickness (Fig. 3A and B), which is a pattern seen in high-grade dysplastic lesions, especially well followed by EGFR (Fig. 5).

IMP3 immunostaining in specimens without atypia was predominantly negative (87.10%), with a scarce positive reaction of weak intensity in single cells or small groups of cells in the superficial layer.

Low-grade dysplasia displayed a predominantly low-intensity staining pattern of IMP3 positivity (55.76%) in the superficial and sometimes suprabasal layer (Fig. 4A and B). No high-grade dysplasia was negative with all five specimens having some IMP3-positive cells.

We recorded an increase of IMP3-positive cells with an increase of atypical changes in laryngeal epithelium, and the chi-square test showed a statistically significant difference of IMP3 IHC staining between the pathohistological groups ($P = 0.003$).

5. Discussion

Adequate management of hoarseness diagnostically as well as therapeutically can sometimes be very challenging. Upon laryngeal examination, often unilateral or bilateral lesions of the vocal cords can

Table 2
Immunohistochemical analysis of markers according to pathohistological classification.

Immunohistochemical markers	Pathohistological classification				
	Normal epithelium / SH	Low-grade dysplasia	High-grade dysplasia	*F*	*P*
EFGR (%) mean ± SD	29.68 ± 8.82	39.96 ± 9.98	56.66 ± 11.0	20.72	< 0.001*
cyclin D1 (%) mean ± SD	18.63 ± 6.24	18.72 ± 9.12	30.92 ± 11.49	6.02	0.005*
p53 (%) mean ± SD	12.35 ± 5.73	12.57 ± 5.28	23.16 ± 3.14	8.72	0.001*
Ki-67 (%) mean ± SD	12.42 ± 4.24	13.79 ± 5.34	20.64 ± 6.62	6.75	0.003*

* indicated significant *P*; SD – standard deviation; SH – simple hyperplasia.

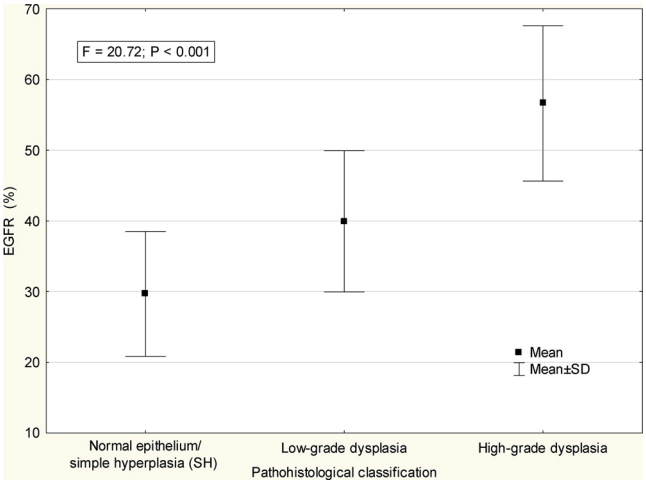

Fig. 1. Percentages of EGFR-positive cell in normal epithelium and SH, low-grade and high-grade dysplasia.

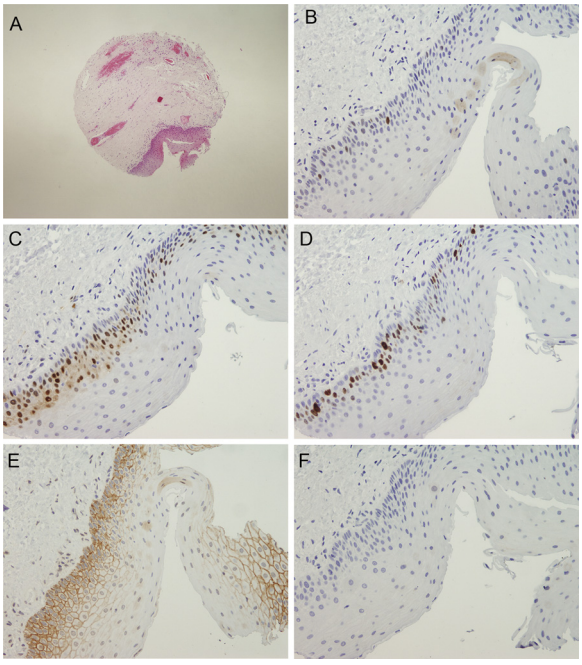

Fig. 2. TMA paraffin sections representing a fibrovascular polypoid formation with hyperplastic epithelium without atypia: A. Haematoxylin-eosin staining (magnification 40x); B. Detail of the epithelium, a clear nuclear immunohistochemical reaction with p53 is shown in some suprabasal cells and rare basal cells, which is in accordance with simple hyperplasia without atypia (magnification x200); C. Immunohistochemical staining with cyclin D1 - nuclear reaction in some suprabasal cells is detected. Only sporadic positive reaction in two cells of the basal layer is noticeable in the visual field (magnification x200); D. Suprabasal and rare basal cells nuclear reaction with Ki-67 immunostaining is detected (magnification x200); E. A strong membranous reaction with EGFR immunostaining in the basal and some suprabasal cells is noticed (magnification x200); F. IMP3 immunostaining on the same tissue, displaying a negative reaction (magnification x200). EGFR indicates epidermal growth factor receptor. IMP3 indicates insulin-like growth factor mRNA-binding protein 3.

be found, ranging from nodal and polypoid formations to a wide range of various forms of exophytic and hyperplastic chronic laryngeal changes, precancerous and tumorous lesions. There is still a lack of consensus in the interpretation of vocal cord changes. This can be attributed to a variety of causes, such as interobserver disagreement and subjective evaluation, institutional differences in terminology and preferences of the specialist in charge [15–18]. Therefore, histological evaluation is mandatory in order to differentiate and properly diagnose these lesions, although vocal nodules and polyps are usually considered as benign formations.

The laryngeal tissue is very active, constantly exposed to microtrauma and noxae, being on the crossroad between respiratory and digestive paths [19,20]. A multitude of factors such as inflammation, repetitive voice burden, especially in certain professions [2], concomitant gastroenterological and rhinological problems and certain treatments (medicamentous, radiological, surgical etc.) can affect the vocal cords apart from well-known noxae, such as tobacco and alcohol [19,21,22]. Laryngeal tissue, having intensive cell renewal and regenerative processes, is a favourable environment for constant epithelial growth and hyperplasia.

One of the theories of carcinogenesis is chronic inflammation [23] and irritation. Atypia can be caused by regenerative processes following inflammation without progression, but carcinogenic downstream is also possible. This led to this investigation of laryngeal carcinogenesis markers on polypoid lesions that are usually considered as regenerative.

Until now, mostly only regenerative factors (such as collagen, fibronectin etc.) [2,24,25] have been studied in detail on vocal nodules and polyps, while parameters of carcinogenesis were only analysed in precancerous lesions.

Having encountered atypia in clinical diagnosis of polyps in our biopsy samples, further research followed and compared architectural and cytologic changes of routine pathohistological sections with molecular markers used in laryngeal carcinogenesis studies.

The level of cell maturity is certainly key in interpreting atypia, but a proper appraisal of the severity of epithelial changes can only be given in the concept of a detailed history and clinical estimation giving us a complete picture of the lesion we are dealing with. Often small bioptic material, and sometimes tangential sections, are tasking even for an experienced pathologist. Multi-focal atypia is especially problematic and these patients can be a challenge to the pathologist and clinician in charge. Nowadays, histopathology alone is not precise

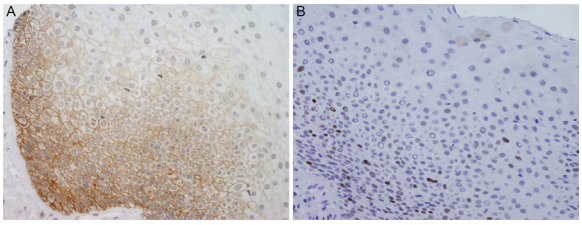

Fig. 3. Immunostaining in fibrovascular polyp with low-grade dysplasia of epithelium (acanthosis, slight nuclear polymorphism, singular mitosis, slight disarrangement of stratification of basal cells): A. membranous EFGR immunostaining: an increased number of suprabasal positive cells in comparison with simple hyperplasia of Fig. 2A is noticed (magnification x200); B. p53 immunostaining displaying sporadic nuclear positivity in basal and suprabasal cells (magnification x200).

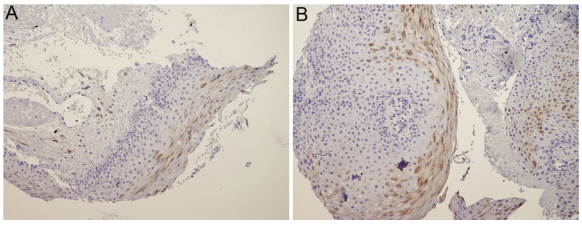

Fig. 4. IMP3 immunostaining: A. Weak cytoplasmic reaction only in the surface layers of fibrovascular polyp (hyperkeratotic and parakeratotic changes and low-grade dysplasia of squamous epithelium) (magnification x100); B. Fibrovascular polyp with partially ulcerated surface displaying stronger changes of epithelium than polypoid tissue in Fig. 2A (acanthosis, nuclear polymorphy, singular mitosis, maintained architectonic of layers). An increased intensity of cytoplasmic IMP3 staining is observed in the suprabasal and superficial layers compared to Fig. 4a which is in accordance with slightly more dysplastic epithelium. No basal cells displaying positive reaction are seen (magnification x100).

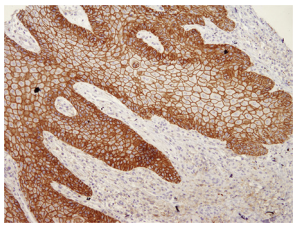

Fig. 5. Clearly shown strong membranous EGFR reaction throughout the epithelial thickness in a fibrovascular polyp showing stronger pathomorphological changes, graded as high-grade dysplasia.

enough in decision making, and besides basic HE slides, one of the key diagnostic tools is IHC analysis. IHC staining of tissue sections is a relatively simple, rapid, reliable and cost-efficient method that can be performed according to standard protocols in many clinical laboratories [26]. Genetic analysis of markers has proven to be much more precise than IHC in the evaluation of precancerous lesions and head and neck cancer (HNSCC), but it is costly and not routinely feasible in most laboratories [27–36].

Molecular studies of various biomarkers have revolutionised our understanding of tumorigenesis providing us with new insights into long-held concepts such as field cancerisation of HNSCC and some markers have been acknowledged the potential application in diagnosis, staging, and prognosis of some cancers [37,38]. Still, there is a lack of information on specific laryngeal tissue, especially laryngeal polypoid formations, because of heterogeneous study groups (often head and neck region) in prior publications of SC carcinogenesis markers [28,29,37] and rare studies on benign formations.

In our study, atypia in polyps was followed by an increase of SC carcinogenesis markers compared to the values of these markers in polypoid tissue displaying normal epithelium or simple hyperplasia as the highest mucosal change. A gradual increase of EGFR, cyclin D1, p53 and Ki-67 protein expression with an increase of dysplasia was observed. Moreover, the study showed a statistically significant correlation between EGFR protein expression and different levels of atypia in hyperplastic lesions. The results also showed a statistically significant difference in cyclin D1, p53 and Ki-67 staining of high-grade dysplastic lesions versus simple hyperplasia and low-grade dysplasia, confirming the need for caution and different treatment of such polypoid lesions which bear a different malignant potential, an increased risk of progression to cancer [39].

Although there is still a lack of standardization in IHC analysis of different markers like EGFR and p53, which sometimes causes difficulty in comparisons of results from different laboratories and authors [40,41], our results were in accordance with literature data [5,8,42,43].

The EGFR staining pattern was in concordance with our extensive previous work on EGFR in laryngeal carcinogenesis [5,6], as membranous EGFR protein expression gradually increased throughout the epithelial thickness with increased atypia. The results support EGFR as one of the most reliable markers of laryngeal dysplasia and literature data that increased EGFR protein expression is associated with biological aggressiveness of glottic precancerous lesions [7,40,42].

IHC analysis of cyclin D1, p53 and Ki-67 showed the staining pattern mainly encountered in normal mucosa of the head and neck region [33,44] with positivity in some suprabasal cells and sporadic staining of basal cells in specimens without atypia. The percentage of positive cells was statistically higher in high-grade dysplastic lesions, and an increase of positive cells towards superficial layers was noticeable for all these markers.

In regard to the usual noxae reported in literature [19,45–47] prolonged vocal abuse and alcohol did not prove to be a noteworthy factor in our study, while GORD was interestingly noticed in 4 out of 5 patients with high-grade dysplasia and positive history of smoking in 10 out of 11 patients with low-grade dysplastic changes. The collected data about smoking and especially drinking were sometimes unconvincing, therefore, in our study, the influence of both noxae on polypoid lesions, is probably unreliable.

Literature data about markers and smoking is often contradictory [48,49]. The strongest connection had been reported about p53 [7,44,50]. In our study, IHC p53 positivity was slightly higher in smokers, but without statistical significance. Worth noticing is that Ogden and coauthors found that smoking increased p53 protein expression among cancer patients but not among healthy individuals [34].

To the best of our knowledge and available literature data, we are the first to report some interesting findings on IMP3 staining. In contrast to the usual staining patterns of molecular markers reported in literature [5,6,8,35,44] that progress with atypia from basal toward superficial epithelial layers, in our research IMP3 staining displayed an inverse progression, showing positivity mainly in superficial and sometimes suprabasal layers, and positivity growing from surface towards basal layers with increase of atypia. We recorded an increase of IMP3-positive cells with an increase of epithelial atypia and all high-grade dysplastic lesions showed a positive IMP3 reaction.

In the only previous study about IMP3 in laryngeal carcinogenesis [10], the authors did not observe IMP3 expression in benign epithelium adjacent to laryngeal carcinoma, mild or moderate dysplasia, or pseudoepitheliomatous hyperplasia, while carcinoma in situ/severe dysplasia was positive for IMP3 in 84% of cases. On the other hand, Di Lu and coauthors [13], researching oesophageal adenocarcinoma, had strong IMP3 positivity in 94% of high-grade oesophageal dysplasia, 14% of low-grade dysplasia and 7% Barrett's oesophagus investigated. Expression of IMP3 was not found in adjacent benign squamous and glandular mucosa and the authors concluded that IMP3 was a highly

sensitive and specific biomarker for the diagnosis of invasive oesophageal adenocarcinoma and high-grade dysplasia. In the research of Li S and coauthors on oral squamous cell carcinomas [11], no data about dysplastic lesions was mentioned. The meaning of our findings in glottic tissue has yet to be studied in future trials. Caution is needed in interpreting IHC staining results. The sensitivity of IMP3 staining is not 100% [10] and methodology not yet routinely performed. Artifacts are possible, as well as false positive and negative results, which in a small initial case series like our preliminary work is not sufficient and conclusive enough for any bold statement and statistical analysis. This is why at this stage, we are only presenting newly noticed staining patterns about IMP3 in laryngeal polypoid tissue, which in our opinion, are worthy of further investigation. Our results indicate the possible use of this marker in an evaluation of laryngeal tissue atypia. In order to obtain relevant conclusions, further research on larger series is ongoing on our clinic and at the pathology department.

Based upon pathohistological findings on our tissue samples, the distinction was made between low-grade and high-grade dysplasia according to the newest classifications of hyperplastic laryngeal lesions (HLL) [14,45,50,51]. While initial mild changes and minimal cellular atypia can be regenerative, lesions that are more atypical have a more malignant potential and demand different treatment [14,39,45,52]. In this work, molecular atypia was in concordance with pathohistological changes, backing up the malignant potential of high-grade dysplastic lesions.

The initial results suggest that in problematic cases, IHC analysis could be indicated along with classic histopathology and clinical evaluation, and of interest as a possible predictor of clinical behavior and prognosis in suspicious glottic polypoid formations. We are aware of the small sample size, especially of high-grade dysplasia in this research, and long-term follow-up studies on a larger series are required.

Stronger expression of SC carcinogenesis markers and molecular atypia should certainly be taken into consideration in diagnosis and such polypoid lesions treated more aggressively. Caution is obligatory, especially in elderly male patients, as high-grade dysplastic lesions were expectedly statistically higher in older patients. Observation is not advisable and patients must be informed that at least biopsy is a must for proper evaluation of the lesion. Patients sometimes do not understand the meaning of their diagnoses and avoid check-up appointments, thinking biopsy is the final cure. It is important to plainly explain the level of aggressiveness and possible risk of the polypoid lesion, and accordingly, the need for further follow-up.

The specialist in charge, as well as the patient, must keep an open mind about what might be indicted in the treatment of different polypoid lesions that lead to dysphonia. We must always consider all other anamnestic data, and according to them, make or alter follow-up decisions for each patient individually, in the context of the entire clinicopathological picture. An older patient with simple hyperplasia or low-grade dysplastic changes which, according to literature follow-up reports [39] have low progression to severity and no malignant transformation, will probably have no significant influence on life expectancy and quality. Therefore, they require less regular controls than younger patients presenting the same pathohistological changes, especially the ones with a voice demanding profession, and smoking or other constant noxae in medical history. On the other hand, in our opinion, careful long-term follow-up, and more frequent controls of polyps with high-grade dysplasia, foremost in younger patients with voice burden history, are recommended.

Evaluation of laryngeal epithelium represents diagnostic challenges, even under normal circumstances. A detailed pathohistological analysis and good feedback and cooperation between pathologists and clinicians is the key to the correct histological and clinical interpretation and further optimal treatment of laryngeal polypoid lesions. We believe that this initial information about markers of SC carcinogenesis in polyps can be an incitement for further research, and perhaps be helpful in proper diagnosis and assessment of aggressiveness, follow-up, and treatment of vocal cord polypoid formations.

We hope that this work can contribute to our position and proper management of polyps, especially when multi-centric foci of atypia are detected.

These are new observations of carcinogenesis markers in hyperplastic glottic lesions included in clinical groups of polyps, in which only markers of regenerative processes were usually analysed. The detected pathohistological and molecular atypia in polypoid changes of vocal cord suggest their inclusion in studies of laryngeal carcinogenesis.

6. Conclusions

Pathohistological analysis detected atypia in some clinically diagnosed polyps.

Further immunohistochemical analysis of these lesions revealed an increase of squamous cell carcinogenesis markers compared to polyps in which simple hyperplasia was the main mucosal change, confirming a correlation of molecular and pathomorphological atypia.

The study confirmed EGFR as one of the most reliable markers in differentiating laryngeal changes and epithelial dysplasia.

Only polyps presenting with simple hyperplasia as the greatest mucosal change can be considered as benign lesions and taken as controls in studies of laryngeal carcinogenesis.

These findings could be incentive for further research of laryngeal carcinogenesis markers and their meaning in fibrovascular polyps.

Careful long-term follow-up of polyps with high-grade dysplasia, especially in younger patients with voice abuse, is recommended.

Declarations of interest

None.

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