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Expression of Periostin in Vocal Fold Polyps

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Long-term voice abuse or sudden vocal fold microvascular disruption may lead to injury and subsequent repair/remodeling in the vocal fold mucosa. Periostin is known to be involved in airway remodeling and also in various otolaryngological diseases. The aim of this article was to investigate the expression and the role of periostin in the formation of vocal fold polyps. The expression patterns of periostin in 59 surgical specimens of vocal fold polyps from 54 patients were investigated immunohistochemically. Normal vocal fold mucosa specimens from 5 patients who had undergone total laryngectomy were used as the control group. Retrospective study with planned data collection was conducted at Tohoku Medical and Pharmaceutical University. Expression of periostin was detected in 43 (72.9%) samples and four patterns of periostin expression were observed in vocal fold polyps: negative type, superficial type, infiltrative type, and diffuse type. An association was observed between periostin expression patterns and the histological subtypes of vocal fold polyps. The infiltrative pattern of periostin expression was significantly dominant in vascular-hyaline types. Expression of transforming growth factor-β (TGF-β) was also detected in the vocal fold polyps. Our results confirmed that periostin might be involved in certain pathological changes in vocal fold polyps, such as extracellular matrix accumulation, local fibrosis, and formation and development of vocal fold polyps.

Keywords: otolaryngological diseases; periostin; remodeling; TGF-β; vocal fold polyp
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Introduction

The presenting symptoms of voice disorders such as vocal fold polyps, laryngeal nodules, laryngeal granulation, and polypoid degeneration include hoarseness and throat discomfort, which not only are responsible for decreasing quality of life but also impede communication in daily life (Martins et al. 2011). Vocal fold polyps are the most common benign lesions of the vocal folds (Kumai 2019). Chronic trauma to the vocal folds from long-term voice abuse, gastric acid reflux, bacteria, or viral pathogens might induce inflammation and tissue repair, contributing to the pathophysiology of vocal fold polyps (White 2019). Recent evidence has suggested that the location of vocal fold fibroblasts in the lamina propria and their ability to respond to external stimuli by producing inflammatory molecules might be involved in the pathophysiology of vocal fold polyps (Naunheim and Carroll 2017; White 2019). Periostin is a matricellular protein and also regulator of fibrosis and collagen deposits, and it has been recognized as a mediator of airway remodeling. The production of periostin was induced by interleukin (IL)-4 and IL-13 dependent manner

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or transforming growth factor-β (TGF-β) dependent manner. Recent studies indicate that periostin plays an important role in the formation of nasal polyps in chronic rhinosinusitis (Ishida et al. 2012). Similarly, there are indications that long-term voice abuse or sudden vocal-fold microvascular disruption may lead to injury and subsequent periostin-mediated repair/remodeling in the vocal fold mucosa. In this study, we investigated the expression and the role of periostin in the formation of vocal fold polyps.

Materials and Methods

Subjects

The patient group consisted of 54 subjects with vocal fold polyps, aged from 25 to 77 years (mean age 51.5 years), who did not respond to at least 3 months' conservative therapy, necessitating surgical removal of the vocal fold polyp(s). All patients underwent removal of vocal fold polyps by endolaryngeal microsurgery at the Division of Otolaryngology, Tohoku Medical and Pharmaceutical University Hospital between April 2015 and November 2019. Forty-nine patients had single lesions and 5 patients had double lesions. Only cases with typical clinical and histological findings were included in the patient group. Clinically, vocal fold polyps were considered as nodular lesions of various sizes which were localized on the free margin or upper surface of the vocal fold and which appeared visually to be a single lesion with a smooth and regular surface, to be grayish-red to dark-red in color, and to have distinct margins surrounded by normal tissue of the vocal fold. The patients with vocal fold polyps were categorized into four histological subtypes: edematous, vascular-hyaline, fibrous, and myxoid respectively. Small blood vessels appearing as hemorrhages sometimes were seen through the thin epithelial layer. Normal vocal fold mucosa specimens from 5 patients aged from 64 to 79 years (mean age 72 years) who had undergone total laryngectomy without radiotherapy were used as the control group. Histopathological diagnosis of vocal fold polyps was made at the Division of Pathology, Tohoku Medical and Pharmaceutical University Hospital. The study was approved by the ethics review committee at Tohoku Medical University Hospital (approval number 2016-2-058) and Tohoku University Graduate School of Medicine (approval number 2017-1-321), and the requirement for informed consent was waived due to the opt-out policy adopted in the study.

Immunohistochemistry to detect periostin, TGF-β, and IL-13

For immunohistochemical detection of periostin, IL-13, and TGF-β, we used the labeled streptavidin-biotin-complex (SABC) method (Ishida et al. 2012; Ohta et al. 2013, 2014). Briefly, 10-μm sections were taken from paraffin-embedded tissue blocks. Deparaffinized tissue sections were rehydrated in a graded series of alcohols. The sections were autoclaved for 10 min at 120°C in citrate phosphate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 30 min. The sections were then incubated with skim milk in phosphate-buffered saline (PBS) for 10 min to block nonspecific background staining. A polyclonal anti-periostin antibody was generated by immunizing rabbits with specific peptides. The anti-periostin polyclonal antibody was kindly provided from Dr. Izuhara as described previously, and applied as a primary antibody at a dilution of 1:500 and incubated at 4°C overnight (Ishida et al. 2012; Ohta et al. 2013, 2014). The anti-TGF-β polyclonal antibody (Invitrogen, Carlsbad, CA, USA) and anti-IL-13 polyclonal antibody (Invitrogen) were also applied as a primary antibody at a dilution of 1:100 or 1:500 respectively. After washing the sections with PBS, biotinylated goat anti-rabbit IgG (Dako Cytomation, Glostrup, Denmark) was applied and the sections were then incubated for 1 hour at room temperature. Histofine MAX-PO(R) (Nichirei Bioscience, Tokyo, Japan) detection reagent with diaminobenzidine substrate was used in accordance with the manufacturer's instructions (Ishida et al. 2012; Ohta et al. 2013, 2014). The specimens from patients with eosinophilic otitis media were used as positive control for IL-13 in the present study (Ohta et al. 2014).

Assessment of immunostained sections

Immunostained sections were assessed at × 40, × 100, and × 200 magnification under a light microscope with an eyepiece reticle. At least two sections were immunostained, and more than five areas were evaluated via the reticle.

Statistics

Variables were compared by Pearson's χ^2 test, Fisher's exact test and Mann-Whitney U test using the statistical software SPSS version 25 (IBM, Chicago, IL, USA). A P-value of less than 0.05 was considered to be statistically significant.

Results

Histological subtypes of vocal fold polyps

The vocal fold polyps were categorized into four histological subtypes. Fig. 1 shows control (Fig. 1A-D) and four histological subtypes; edematous (Fig. 1E-H), vascular-hyaline (Fig. 1I-L), fibrous (Fig. 1M-P), and myxoid (Fig. 1Q-T). The clinical information of the patients was demonstrated in Table 1. The edematous type is characterized by submucosal accumulation of pale blue- to pink-stained material admixed with a sparsely cellular and variably vascularized stroma (Fig. 1F, G). The vascular-hyaline type is characterized by dense eosinophilic submucosal deposition of fibrin material, often in close proximity to a vascular space (Fig. 1J, K). The fibrous type is characterized by moderately cellular submucosa consisting of uniform oval to spindle-shaped cells associated with a varying amount of fibrous tissue deposition (Fig. 1N, O). The myxoid type is marked by prominent dilated vascular spaces

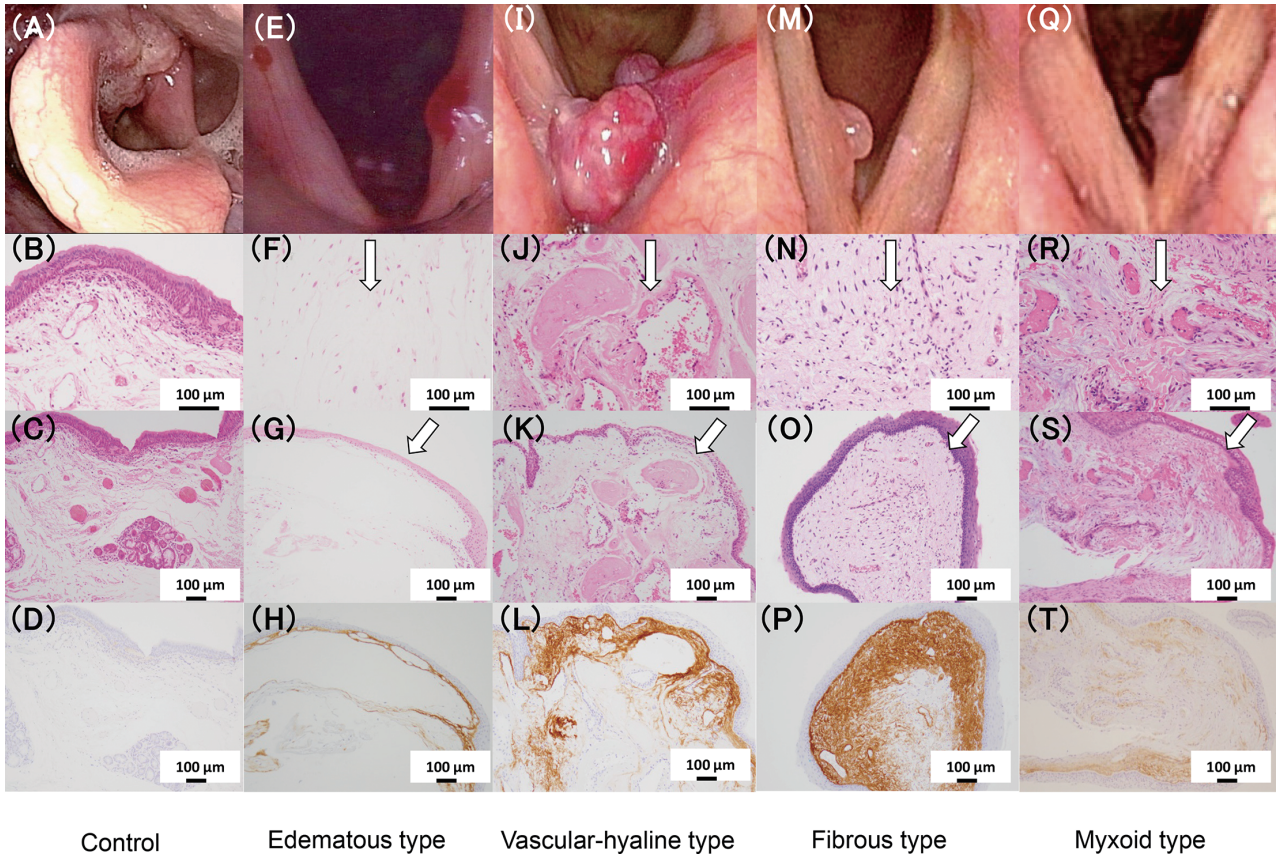

Fig. 1. Laryngeal findings, hematoxylin and eosin (HE) staining and immunohistochemical staining of periostin in control and vocal fold polyps.
(A) Case 1. A hypopharyngeal mass is observed in the right pyriform sinus. Normal vocal fold mucosa specimen was used. (B, C) Mucosal epithelium and lamina propria (HE staining, original magnification × 200 and × 40, respectively). (D) Expression of periostin was not detected (immunostaining, original magnification × 40). (E) Case 2. (F, G) This case is a typical edematous type. Submucosal accumulation of pale blue- to pink-stained material admixed with a sparsely cellular and variably vascularized stroma is observed (arrow) (HE staining, original magnification × 200 and × 100, respectively). (H) Periostin is mainly observed in the subepithelial layers between the basement membrane and the stromal tissue (immunostaining, original magnification ×100). (I) Case 3. (J, K) This case is a typical vascular-hyaline type. Dense eosinophilic submucosal deposition of fibrin material closely apposed to a vascular space is observed (arrow) (HE staining, original magnification × 200 and × 100, respectively). (L) Periostin is observed from the basement membrane to the lamina propria in varying degrees (immunostaining, original magnification × 100). (M) Case 4. (N, O) This case is a typical fibrous type. Moderately cellular submucosa consisting of uniform oval to spindle-shaped cells associated with a varying amount of fibrous tissue deposition is observed (arrow) (HE staining, original magnification × 200 and × 100, respectively). (P) Periostin is expressed throughout the lamina propria starting from just below the basement membrane (immunostaining, original magnification × 100). (Q) Case 5. (R, S) This case is a typical myxoid type. Markedly prominent dilated vascular spaces with or without associated hemorrhage and a combination of the above subtypes in a single lesion is observed (arrow) (HE staining, original magnification × 200 and × 100, respectively). (T) Periostin is observed from the basement membrane to the lamina propria in varying degrees (immunostaining, original magnification ×100).

with or without associated hemorrhage and a combination of the above subtypes in a single lesion (Fig. 1R, S). Vascular-hyaline and edematous types were the most common among the four types (Table 2).

Expression of periostin in vocal fold polyps

The expression of periostin was investigated in 59 samples of vocal fold polyps from 54 patients (Table 2). The expression of periostin was detected in 43 (72.9%) samples obtained from patients with vocal fold polyps. Four types periostin expression were observed in vocal fold polyps: negative (Fig. 2A-C), superficial (Fig. 2D-F), infiltrative (Fig. 2G-I), and diffuse (Fig. 2J-L). In the superficial type of periostin expression, periostin is detected only in the subepithelial layers between the basement membrane and the stromal tissue (Fig. 2F). In the infiltrative type, periostin is observed from the basement membrane to the lamina propria in varying degrees (Fig. 2I) and in the diffuse type, periostin is expressed throughout the lamina propria starting from just below the basement membrane (Fig.

Table 1. Clinical information of the patients.

Case	Sex	Age	Diseases	Type	Periostin	Figure
1	Male	69	Hypopharyngeal cancer	control	negative	Fig. 1A-D
2	Female	56	Laryngeal polyps	edematous	positive	Fig. 1E-H
3	Male	50	Laryngeal Polyps	vascular-hyaline	positive	Fig. 1I-L
4	Male	61	Laryngeal Polyps	fibrous	positive	Fig. 1M-P
5	Male	26	Laryngeal Polyps	myxoid	positive	Fig. 1Q-T

Table 2. Association of periostin expression patterns and histological subtypes of vocal cord polyps.

	Negative type	Superficial type	Infiltrate type	Diffuse type
Edematous type (n = 20)	5	8	6	1
Vascular-hyaline type (n = 23)	7	2	14	0
Fibrous type (n = 14)	4	2	4	4
Myxoid type (n = 2)	0	0	2	0
Total 59	16 (27.1%)	12 (20.3%)	26 (44.1%)	5 (8.5%)

Data are presented as n or n (%). Pearson's x^2 =1.233; P = 0.016.

2L). Various levels and types of periostin expression were observed in all histological subtypes of vocal fold polyps.

Expression of IL-13 and TGF-β in vocal fold polyps

The expression of periostin was detected in 43 (72.9%) samples obtained from patients with vocal fold polyps. The expression of TGF-*β* was detected in all periostin positive samples, however the expression of IL-13 was not observed in same samples. The expressions of IL-13 and TGF-*β* were not observed in periostin negative cases (data not shown).

A representative case of vocal fold polyps in an elderly Japanese man is presented (Fig. 3). This patient is a representative case of vascular-hyaline type and infiltrative type. Strong staining for periostin was observed in laryngeal tissue from the patient (Fig. 3B). IL-13-positive infiltrating cells were not detected in laryngeal tissue (Fig. 3C), however, TGF-*β*-positive infiltrating cells were detected (Fig. 3D).

Expression of periostin and clinicohistological factors

An association was observed between periostin expression patterns and the histological subtypes of vocal fold polyps. The superficial pattern was the most common in the edematous type of vocal fold polyps. The diffuse pattern was also the most common in the fibrous type of vocal fold polyps. The infiltrative pattern was significantly dominant in the vascular-hyaline type of vocal fold polyps (Table 2). No significant relationship between periostin expression pattern and clinical characteristics including comorbidities of allergic disease, smoking, intubation, or periods of illness was observed (Table 3).

Discussion

Various levels and patterns of periostin expression were observed in all histological subtypes of vocal fold polyps. Microscopic examination revealed that hemorrhage, fibrin exudation, fibrosis, proliferation of capillaries, and dilated vascular spaces were found in the vocal fold polyps. The appearance of these findings varied among the edematous, vascular-hyaline, fibrous, and myxoid types of vocal fold polyps.

It might be suggested that the disease etiology of vocal fold polyps involves the injury of vessels leading to repair/remodeling as follows. Often the first step to vocal fold polyps is long-term voice abuse or sudden vocal fold microvascular disruption due to phonotrauma (brusque movements of the vocal fold membrane during phonation). It could be possible that the latter leads to the injury of the vessels, and thus to hemorrhage, leakage of fibrin, thrombosis, proliferation of capillaries, hyalinization, and fibrosis. This pathology can also be caused by aggravated tension of the vocal folds or by other factors (such as infection, dust, alcohol, smoking, or endocrine dysfunction), which affect the mucosa of the vocal folds, thus damaging the walls of blood vessels. The histologic changes might represent different tissue reactions to the initiating event and not progressive or sequential changes.

It is considered that the pathological processes of vocal fold polyps occur mainly in Reinke's space, an area lying deep to the true vocal cords, which is essentially lacking in blood vessels and which in response to stimuli has a tendency to accumulate fluid. Previous studies report that extracellular matrix proteins, including collagenase, elas-

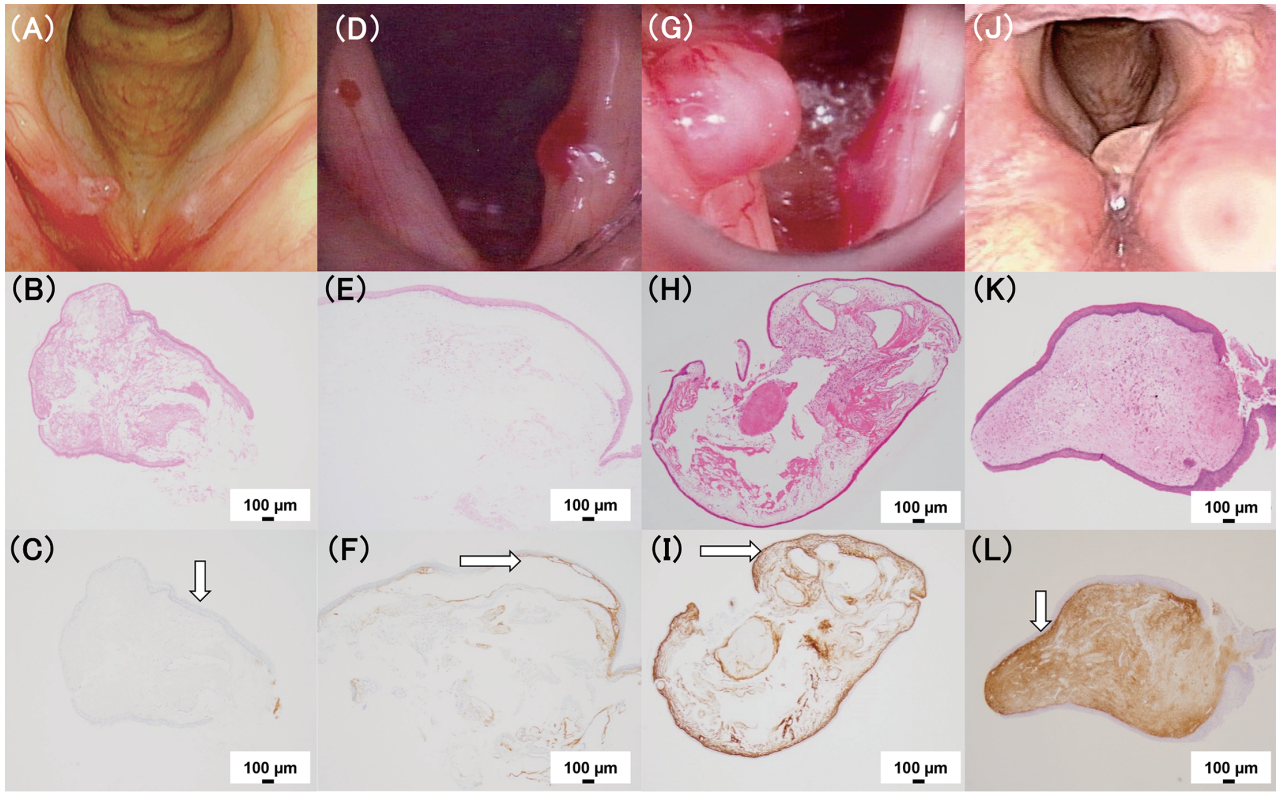

Fig. 2. Immunohistochemical staining patterns of periostin in vocal fold polyps, which were categorized into four types: negative type (n = 16), superficial type (n = 12), infiltrative type (n = 26), and diffuse type (n = 5) (Original magnification × 40).
(A) A 46-year-old Japanese male with polyps on bilateral vocal folds. (B) Dense eosinophilic submucosal deposition of fibrin material closely apposed to a vascular space is observed. The diagnosis of vascular-hyaline type was made (HE staining). (C) Periostin is not observed: Negative type (arrow) (immunostaining). (D) A 56-year-old Japanese female with red polyps on bilateral vocal folds. (E) Submucosal accumulation of pale blue- to pink-stained material admixed with a sparsely cellular and variably vascularized stroma is observed, and the diagnosis of edematous type was made (HE staining). (F) Periostin is detected only in the subepithelial layers between the basement membrane and the stromal tissue; superficial type (arrow) (immunostaining). (G) A 42-year-old Japanese female with red polyps on bilateral vocal folds. (H) Dense eosinophilic submucosal deposition of fibrin material closely apposed to a vascular space was observed, and the diagnosis of vascular-hyaline type was made (HE staining). (I) Periostin is observed from the basement membrane to the lamina propria in varying degrees, and the diagnosis of infiltrative type was made (arrow) (immunostaining). (J) A 67-year-old Japanese male with a white polyp on the left vocal fold. (K) Moderately cellular submucosa consisting of uniform oval to spindle-shaped cells associated with a varying amount of fibrous tissue deposition is observed, and the diagnosis of fibrous type was made (HE staining). (L) Periostin is expressed throughout the lamina propria starting from just below the basement membrane, and the diagnosis of diffuse type was made (arrow) (immunostaining).

tase, fibronectin, fibromodulin, decorin, hyaluronic acid synthase 2, and hyaluronidase —all of which are involved in tissue repair/remodeling— might contribute to the formation of vocal cord polyps (Martins et al. 2011; Cipriani et al. 2011; Ishida et al. 2012; Fang et al. 2013; Ohta et al. 2013, 2014; Uloza et al. 2015; Naunheim and Carroll 2017; Kirgezen et al. 2017; Wang et al. 2017; Hamdan et al. 2018; Kumai 2019; White 2019; Liutkevicius et al. 2020).

Recent studies have demonstrated that periostin might be involved in different ways in various otolaryngological diseases, including allergic rhinitis, chronic rhinosinusitis with nasal polyps, organized hematoma, eosinophilic otitis media, and IgG4-related diseases (Ishida et al. 2012; Ohta et al. 2013, 2014; Akdogan et al. 2015). However, the role of periostin in vocal fold polyps has not been reported.

Periostin is characterized not only by its role as an extracellular matrix structural protein which regulates fibrosis and collagen deposition, but also by its role as a matricellular protein, which regulates the Th2-mediated inflammation cascade (Ishida et al. 2012; Ohta et al. 2014; Shiono et al. 2015). Shiono et al. (2015) reported that in nasal polyps associated with chronic rhinosinusitis there were two different patterns of periostin expression - “superficial type” and “diffuse type” - and the diffuse pattern of periostin expression was highly correlated with the number of infiltrating eosinophils. In the present study, four patterns of

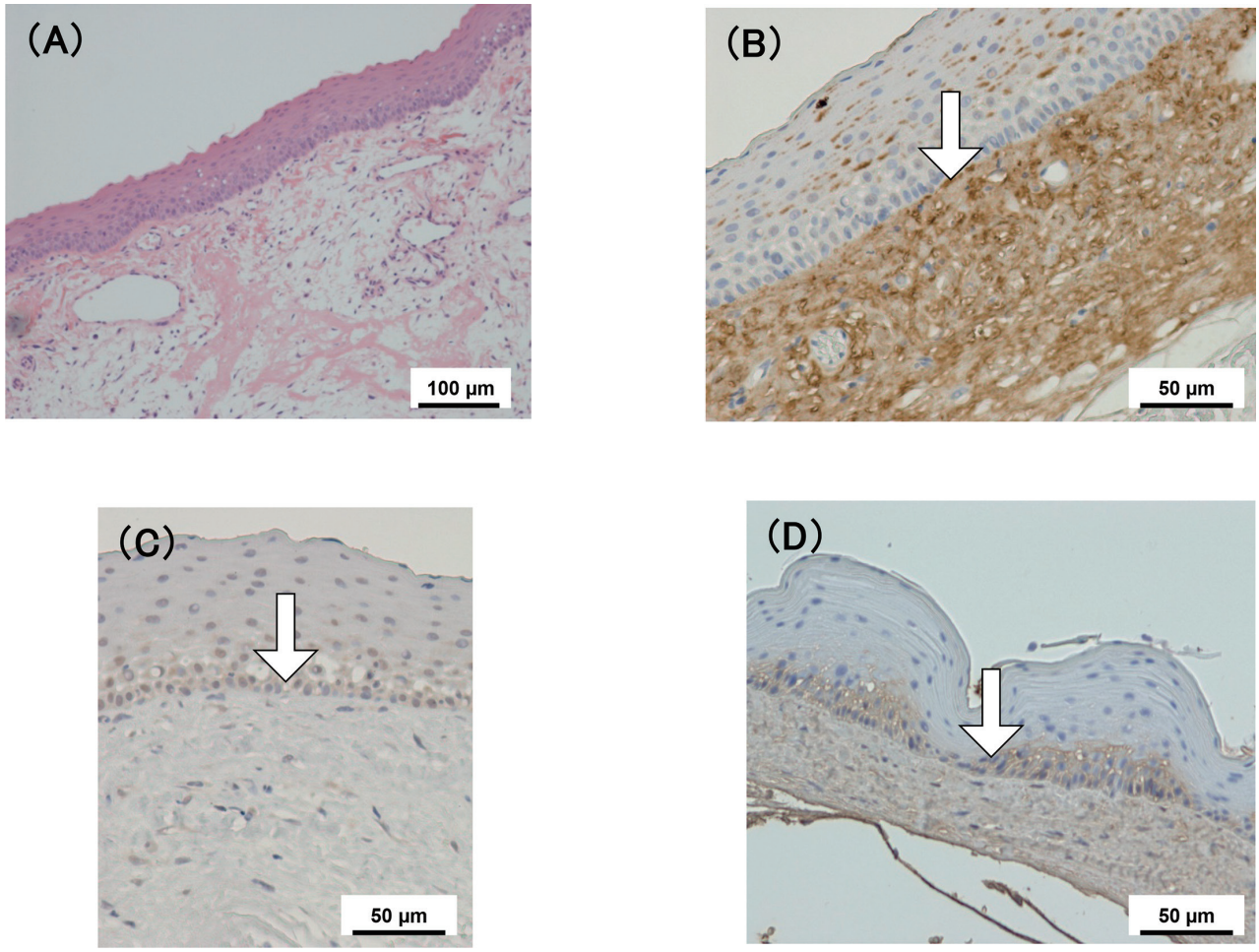

Fig. 3. Immunohistochemical staining of IL-13 and TGF-β in a 69-year-old Japanese male as a representative case.
(A) Infiltration of hyalinization and vascular components is observed in laryngeal tissue obtained from the patient (HE staining, original magnification × 200). (B) Strong staining for periostin is observed in laryngeal tissue (arrow) (× 160). (C) IL-13-positive infiltrating cells were not detected in laryngeal tissue (arrow) (× 400). (D) TGF-β-positive infiltrating cells were detected in laryngeal tissue (arrow) (× 400).

periostin expression were observed in vocal fold polyps: negative type, superficial type, infiltrative type, and diffuse type. The infiltrative pattern of expression was significantly more common in vascular-hyaline type polyps than in the other types. These findings may allow us to speculate an impact of periostin on remodeling and neovascularization in stroma tissue of vascular-hyaline type vocal fold polyp. There is also increasing evidence that not only fibroblasts and airway epithelial cells but also microvascular endothelial cells produce periostin (Ishida et al. 2012; Ohta et al. 2013; Shiono et al.2015). It is generally accepted that the expression of periostin is induced by IL-4 and IL-13 (Ohta et al. 2014). However, contrary to our expectation, IL-13 was not detected in affected laryngeal tissues from patients with vocal fold polyps. In lung tissues, periostin increases the synthesis and secretion of collagen and promotes epithelial-mesenchymal transition in a TGF-β-dependent manner (Ohta et al. 2013, 2014). There are presently two known pathways to induce periostin: in an IL-13-/IL-4-dependent manner and in a TGF-β-dependent manner (Ohta et al. 2013, 2014). Our results clearly demonstrate that periostin might be involved in the pathogenesis of vocal fold polyps in a TGF-β-dependent manner. Limitation of the present study is that the expression of periostin should be estimated by not only immunohistochemical analysis but also western blotting and RT-PCR multilaterally. However, the surgical specimens obtained from patients with laryngeal poly(s) were quite small to investigate with western blotting and RT-PCR. Previous study demonstrated that the expressions of periostin in nasal mucoma obtained from patients with normal subjects, allergic rhinitis, and chronic rhinosinusitis with nasal polyps were relatively correlated with western blotting and RT-PCR data (Ishida et al. 2012). Another limitation of the preset study is that the number of patients with laryngeal polyps and the surgical specimen obtained from patients were limited in the present case to compare the patient characteristics and pathological findings between positive type and negative type respectively. Further study will be warranted to explore these points.

Periostin is transiently upregulated after mechanical

Table 3. Clinical characteristics and expression of periostin in 59 samples of vocal fold polyps from 54 patients.

Characteristics of factor	Total (n = 54)	Periostin negative (n = 14)	Periostin positive (n = 41)			P-value
		Negative type (n = 14)	Superficial type (n = 12)	Infiltrate type (n = 24)	Diffuse type (n = 5)	
Sex						0.564
Male, n (%)	30 (55.5%)	8 (57%)	5 (12%)	14 (34%)	4 (10%)	
Female, n (%)	24 (44.4%)	6 (43%)	7 (17%)	10 (24%)	1 (2%)	
Mean age (years) mean ± SD (range)	51.5 ± 14.2 (25-77)	48.9 ±14.5 (30-75)	46.9 ± 12.3 (29-69)	54.7 ± 15.2 (25-77)	59.6 ± 7.5 (48-67)	0.536
Period of illness (month) mean ± SD	17.7 ± 34.3	11.1 ± 17.4	15.4 ±19.3	21.0 ± 45.6	16.2 ± 25.2	0.846
Intubation, n (%)	6 (11%)	2 (14%)	2(5%)	2 (5%)	0 (0%)	0.060
Tabacco, n (%)	#37 (69%)	#9 (64%)	8 (20%)	#18 (44%)	3 (7%)	0.896
Allergy, n (%)	23 (43%)	7 (50%)	4 (10%)	10 (24%)	2 (5%)	0.861
Bronchial asthma, n (%)	6 (11%)	0 (0%)	1 (2%)	5 (12%)	0 (0%)	0.109
Nasal allergy, n (%)	11 (20%)	5 (36%)	3 (7%)	3 (7%)	0 (0%)	0.137
Food allergy, n (%)	5 (9%)	2 (14%)	0 (0%)	3 (7%)	0 (0%)	0.546
Allergic conjunctivitis, n (%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0.110
Drug allergy, n (%)	3 (6%)	1 (7%)	1 (2%)	0 (0%)	1 (2%)	0.272
Allergic dermatitis, n (%)	2 (4%)	1 (7%)	0 (0%)	1 (2%)	0 (0%)	0.811
Other allergy, n (%)	3 (6%)	1 (7%)	0 (0%)	2 (5%)	0 (0%)	0.739

#One patient in this group had double lesions, which one lesion was periostin negative and another lesion was periostin positive.

stress and injury under physiological conditions (Ohta et al. 2013). This process is vital for normal wound healing. On the other hand, continuous and longstanding overexpression of periostin can lead to persistent, slowly progressive inflammation with remodeling and restructuring (Ohta et al. 2014). There is thus a process of transition from local, normal expression of periostin to expression under pathological conditions. Proper treatment might decrease periostin expression and its newly recognized functions in inflammatory reactions and remodeling, with clinical implications for wound healing and tissue repair in laryngeal tissues. Periostin might therefore be useful as an additional biomarker of disease activity and may provide brand-new therapeutic targets for preventing pathological processes involved in vocal fold polyps.

In conclusion, our results indicate that overexpression of periostin is likely involved in the pathogenesis of vocal fold polyps, and we speculate that periostin could be a novel biomarker for, and a therapeutic target in, vocal fold polyps.

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Conflicts of Interest

The authors declare no conflict of interest.

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