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The Role of Keratin-8 and Keratin-18 Polymorphisms and Protein Levels in the Occurrence and Progression of Vocal Leukoplakia

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Keywords
Keratin-8 · Keratin-18 · Vocal leukoplakia · Polymorphism · Protein level

Abstract
Objective: This study aimed to evaluate the association between the single-nucleotide polymorphism (SNP) and tissue protein level of keratin-8/18 and the occurrence and progression of vocal leukoplakia. ***Methods:*** The case-control study enrolled 158 patients with vocal leukoplakia, 326 patients with laryngeal squamous cell carcinoma (LSCC), and 268 healthy controls, which were tested for genotype analysis with keratin-8 and keratin-18 gene polymorphisms using pyrosequencing. The tissue protein expression levels of keratin-8 and keratin-18 were evaluated using immunohistochemistry. ***Results:*** The keratin-8 SNP RS1907671 showed an obvious increased risk for vocal leukoplakia (OR 1.56, $p = 0.002$), while the other SNPs (RS2035875, RS2035878, RS4300473) were tested as protective factors for vocal leukoplakia and LSCC (OR <1, $p < 0.05$). In keratin-18 SNP test, both RS2070876 and RS2638526 polymorphisms demonstrated decreased risks for vocal leukoplakia and LSCC (OR < 1, $p < 0.05$). The protein levels of keratin-8 and keratin-18 in vocal leukoplakia group were significantly higher than those of the LSCC group ($p < 0.05$). ***Conclusions:*** Keratin-8 and keratin-18 polymorphisms and protein levels are associated with the occurrence and progression of vocal leukoplakia.

Introduction

Vocal leukoplakia is a clinically common precancerous disease of laryngeal squamous cell carcinoma (LSCC), which was characterized with epithelial keratosis and hyperplasia. The malignant transformation rate of vocal leukoplakia to LSCC was evaluated as 14–33% [1–3], so surgical excision was the main treatment strategy for vocal leukoplakia.

The pathogenesis of vocal leukoplakia was not verified, but voice abuse, tobacco and alcohol consumption were often considered as risk factors, and laryngopharyngeal reflux and vitamin deficiency were also deemed as possible factors. In addition to this, many other potential pathogenic factors were also detected. Due to the close relationship with cell morphology, motility and differen-

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tiation, and angiogenesis and metastasis in carcinoma progression [4], extracellular matrix protein 1 (ECM-1) was detected as a novel research point in solid tumors [5, 6]. In the study organized by Gu et al. [7], the ECM-1 was indicated as a crucial point in the cancerization of vocal leukoplakia to LSCC. Some recent studies put the emphasis on genetic changes of vocal leukoplakia, such as matrix metalloproteinase, DNA ploidy, P16, B-cell lymphoma/leukemia 11A and TP53, which showed risk for the progression of vocal leukoplakia to LSCC [8–11]. However, the study focus on the keratins for the occurrence and progression of vocal leukoplakia was rare.

Keratins are well known as the largest family of intermediate filament-forming proteins, which are mainly expressed in epithelial cells [12, 13]. The keratins are commonly named as keratin-1 to keratin-20, which are oligomerized to be heterodimer partners to form filaments by non-covalent coiled coil interaction between one type I keratin (keratin-9 to keratin-20) and one type II keratin (keratin-1 to keratin-8). The formed intermediate filaments support the cell integrity and continuity by providing the shape and rigidity of the cells. In addition to this, the keratins also play an important role in cell apoptosis, membrane transportation, cellular proteins synthesis and transportation, and stress response of cells [14–16]. The keratin heterodimer pairs express differently in stratified epithelium. In the basal cell differentiation, the keratin-4/13 pair expresses in internal epithelium and keratin-1/10 pair expresses in cornified epithelium, while the breast epithelium commonly express keratin-5/14 pair in proliferation compartment and keratin-8/18 pair in differentiation compartment [17, 18]. These specific expression patterns of keratin pairs imply that the keratins may have organ- or tissue-special functions.

The keratin-8/18 heterodimer is the first keratin pair to be expressed in embryogenesis, which regulates multiple cellular function, including cell apoptosis, protein localization, and targeting modulation. The aberrant expression of keratin-8/18 pair has been detected in some squamous cell carcinomas and adenocarcinomas [18–21]. Furthermore, the keratin-8/18 expression was observed to be associated with malignant transformation, neoplastic progression, and adverse prognosis [21–23]. Without keratin-18, keratin-8 is confirmed to be relevant to tumor progression in many tumors. For instance, the decreased expression of keratin-8 is detected in the colorectal and breast tumor [24, 25], while an increased expression of keratin-8 is observed in the head and neck [26], digestive tract [27], and oral cavity carcinoma and to be associated with poor prognosis [21]. Some single point mutations of keratin-8 were reported to be relevant with the exchange of amino acids, such as the exchange of a glycine to a cysteine residue and tyrosine to histidine, resulting the cryptogenic liver diseases [28, 29]. Therefore, the keratin-8 and keratin-18 are prone to promote the transformation from normal mucosal tissue to malignant tumor. However, as an important precancerous lesion of LSCC, the study in pathogenesis and disease progression of vocal leukoplakia is still deficient.

Although in recent years there have been many studies on the relationship between mucosal leukoplasia and keratin-8/18, they mostly focused on the oral leukoplasia disease, and generally emphasized the protein level only. Therefore, in the present study, we investigated both the single-nucleotide polymorphisms (SNPs) in gene level and the protein expression in protein level. To our knowledge, this is the first study focused on gene and protein levels to investigate the effects of keratin-8 and keratin-18 on the susceptibility and severity of vocal leukoplakia.

Material and Methods

Ethical Approval

The study protocol was approved by the Medical Research Council of our hospital (No. KJ2008-01) and informed consent was obtained from all patients and healthy (control) individuals. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional and National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Patients and Controls

From September 2014 to June 2017, 158 patients with vocal leukoplakia and 326 patients with LSCC were enrolled in the study from our hospital. As a control group, 268 cases of healthy volunteers with equivalent gender, age, and ethnicity were enrolled in the present study. All cases were detected with a genotype test.

In the immunohistochemistry (IHC) test, a total of 109 vocal leukoplakia patients and 14 LSCC patients with the glottic type were chosen as vocal leukoplakia group and LSCC group, and 31 cases of vocal polyp patients were collected as control group. All samples of patients were confirmed by histopathology. In the vocal leukoplakia group, different dysplasia degrees were divided into 2 subgroups: low risk group (including hyperplasia, mild dysplasia, and moderate dysplasia) and high-risk group (including severe dysplasia and small area with suspected carcinoma cells).

In all groups, smoking consumption was classified as nonsmoker (<100 cigarettes in their lifetime) and smoker (more than 20 cigarettes per day for 1 year or more). Alcohol consumption was classified as non-drinker (<200 mL per day) and drinker (more than 200 mL per day). The clinicopathological characteristics of different subgroups are shown in Table 1.

Table 1. Distribution of cases and controls according to selected sociodemographic characteristics

Characteristics	Controls	Vocal leukoplakia	LSCC	Odds ratio (95% CI)[1]	*p* value[1]	Odds ratio (95% CI)[2]	*p* value[2]
Age, years	61.23±7.6	58.46±9.8	60.88±8.6	–	–	–	–
Gender							
Female	8	5	8	–	–	–	–
Male	260	153	318				
Smoking							
No	187	53	92	Ref.		Ref.	
Yes	81	105	234	4.57 (3.0–7.0)	<0.01	5.87 (4.1–8.4)	<0.01
Alcohol consumption							
No	205	68	123	Ref.		Ref.	
Yes	63	90	203	4.31 (2.8–6.6)	<0.01	5.37 (3.7–7.7)	<0.01
Typing of LSCC							
Glottic type			215				
Supraglottic type			108				
Subglottic type			3				
Stage of LSCC							
Advanced							
III+IV			114				
Initial							
I+II			212				
Lymph node							
N_0			244				
N_1+N_2			82				

[1] OR, *p* value calculated between vocal leukoplakia and controls with SPSS; [2] OR, *p* value calculated between LSCC and controls with SPSS.

Blood Collection and Plasma and DNA Extraction

The peripheral blood with 5 mL of each participant was collected, and then the blood was processed for plasma collection. The plasma was stored at –80 °C within 30 min after collection. Approximately 100 ng/μL of genomic DNA was prepared from the peripheral blood using a QIAamp DNA blood mini kit (QIAGEN Inc., Valencia, CA, USA).

Analysis of Keratin-8 and Keratin-18 SNPs

Keratin-8 and keratin-18 SNPs were analyzed by polymerase chain reaction (PCR) amplification using specifically designed pairs of oligonucleotide primers followed by direct sequencing (ABI Prism 3730xl DNA sequencer, PE Biosystems, Foster City, CA, USA). For keratin-8 and keratin-18 genotyping, the PCR conditions were as follows: 30 cycles at 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 1 min. All laboratory assays were conducted and interpreted blindly without the knowledge of the case or control status. The primer sequences used in the present study were as follows:

Keratin-8:
RS1907671F: 5′ GCCCAATGTTTGCTGAAT 3′
RS1907671R: 5′ GCCAGCCATCTATTTCTT 3′ 420 bp;
RS2035875F: 5′ CCAATCACAAGGTCAGGA 3′
RS2035875R: 5′ GCACTTCCCACAACAGAG 3′ 870 bp;
RS2035878F: 5′ TCTCCCTTCTCCTATCTG 3′
RS2035878R: 5′ TCTGTGAAATGGGGTAAT 3′ 779 bp;
RS4300473F: 5′ TCCTCTATTGGTATTTGCTA 3′
RS4300473R: 5′ AAAGGGACTTGTGATTGG 3′ 858 bp.
Keratin-18:
RS2070876F: 5′ TGCCAAGAATGGTGCTCA 3′
RS2070876R: 5′ CTCCCCTTCACAAACCTG 3′ 653 bp;
RS2638526F: 5′ GGCAGTCAGGGTGGAAGC 3′
RS2638526R: 5′ GGTGGGGCAGGAATCAGA 3′ 677 bp.

IHC Staining and Evaluation

Collected specimens were embedded in paraffin and sectioned as 4 μm slides. The paraffin-embedded sections were deparaffinized in xylene and rehydrated by passing through series of 100, 95, 75, and 50% ethanol followed by water. Endogenous peroxidase activity was inhibited by incubating the tissues in 0.3% H_2O_2 for 10 min at room temperature. After blocking with 5% normal goat serum diluted in phosphate buffered saline, the sections were incubated with 1:100 dilution of primary rabbit anti-human keratin-8 antibody (GB13231, Google Biotechnology Inc., China) or 1:1,000 dilution of primary rabbit anti-human keratin-18 antibody (GB11232, Google biotechnology) at room temperature for 2 h.

Table 2. The association between keratin-8 genotypes and development of controls, vocal leukoplakia, and LSCC

Genotype	Controls (n = 268)	Vocal leukoplakia			LSCC		
		n = 158	OR (95% CI)	p value	n = 326	OR (95% CI)	p value
RS1907671							
AA	140	50	Ref.		142	Ref.	
AC	84	82	2.73 (1.75–4.26)	<0.01	152	1.84 (1.25–2.54)	<0.01
CC	44	26	1.66 (0.92–2.96)	0.09	32	0.72 (0.43–1.20)	0.20
Alleles							
A	364	182	Ref.		436	Ref.	
C	172	134	1.56 (1.17–2.08)	0.002	216	1.05 (0.82–1.34)	0.70
RS2035875							
CC	86	54	Ref.		155	Ref.	
CT	98	46	0.75 (0.46–1.22)	0.24	85	0.48 (0.33–0.71)	<0.01
TT	84	58	1.10 (0.68–1.77)	0.70	86	0.57 (0.38–0.85)	<0.01
Alleles							
C	270	154	Ref.		395	Ref.	
T	266	162	1.07 (0.81–1.41)	0.64	257	0.66 (0.52–0.83)	<0.01
RS2035878							
AA	26	28	Ref.		38	Ref.	
AG	117	73	0.58 (0.32–1.07)	0.08	135	0.79 (0.45–1.38)	0.41
GG	125	57	0.42 (0.23–0.79)	0.006	153	0.84 (0.48–1.45)	0.53
Alleles							
A	169	129	Ref.		211	Ref.	
G	367	187	0.67 (0.50–0.89)	0.006	441	0.96 (0.75–1.23)	0.76
RS4300473							
CC	24	34	Ref.		44	Ref.	
CG	123	69	0.40 (0.22–0.72)	0.002	131	0.58 (0.33–1.01)	0.054
GG	121	55	0.32 (0.17–0.59)	<0.01	151	0.68 (0.39–1.18)	0.17
Alleles							
C	171	137	Ref.		219	Ref.	
G	365	179	0.61 (0.46–0.82)	<0.01	433	0.93 (0.73–1.18)	0.54

After washing, rabbit peroxidase-conjugated second antibody was allowed to bind at room temperature for 1 h. For all slides, the staining of keratin-8 and keratin-18 was visualized with 3, 3′-diaminobenzidine followed by counterstaining with Meyer hematoxylin, dehydrated, and mounted.

Images of the IHC staining were evaluated and scored by an experienced pathologist following the Formwitz Scoring System [30]. Briefly, the density of the brown color and number of positive cells were evaluated or counted in 5 randomly high-power fields and then averaged. The density of brown color of the cells was assessed in 4 degrees: 0 point equals negative staining, 1 point equals weak brown staining, 2 points equals brown staining, and 3 points equals dark brown staining; and the positivity rate of cells was also assessed in 4 groups: 1 point equals positivity rate <25%, 2 points equals positivity rate in 25–50%, 3 points equals positivity rate in 51–75%, and 4 points equals positivity rate >75%. By multiplying the scores of brown-colored density and positivity rate of cell, the IHC staining total scores were evaluated in 4 groups: 0 point equals negative staining, 1–4 points equals weakly positive staining, 5–8 points equals positive staining, and 9–12 points equals strongly positive staining.

Statistical Analysis

The demographic and oncological characteristics of patients, the environmental factors and the gene frequencies of keratin-8 and keratin-18 in all cases were compared and analyzed by χ^2 tests. The differences of IHC staining scores between groups were analyzed by one-way ANOVA with SPSS, and $p < 0.05$ is considered statistically different. The SPSS statistical package was used to analyze the data and 95% CIs and odds ratios (ORs) are presented.

Results

Demographic and Oncological Details

The demographic and oncological characteristics of patients and risk factors in controls, patients with vocal

Table 3. The association between keratin-18 genotypes and development of controls, vocal leukoplakia, and LSCC

Genotype	Controls (n = 266)	Vocal leukoplakia			LSCC		
		n = 155	OR (95% CI)	p value	n = 323	OR (95% CI)	p value
RS2070876							
CC	25	22	Ref.		44	Ref.	
CT	133	79	0.68 (0.36–1.28)	0.225	130	0.56 (0.32–0.96)	0.034
TT	108	54	0.57 (0.29–1.10)	0.091	149	0.78 (0.45–1.36)	0.385
Alleles							
C	183	123	Ref.		218	Ref.	
T	349	187	0.80 (0.60–1.07)	0.125	428	1.03 (0.81–1.31)	0.814
RS2638526							
CC	17	23	Ref.		55	Ref.	
CT	137	67	0.36 (0.18–0.72)	0.003	125	0.28 (0.16–0.51)	<0.01
TT	112	65	0.43 (0.21–0.86)	0.016	143	0.40 (0.22–0.72)	0.002
Alleles							
C	171	113	Ref.		235	Ref.	
T	361	197	0.83 (0.62–1.11)	0.202	411	0.83 (0.65–1.06)	0.128

leukoplakia and LSCC are presented in Table 1. There was no difference in the age of controls (61.23 ± 7.6 year), vocal leukoplakia patients (58.46 ± 9.8 year), and LSCC patients (60.88 ± 8.6 year). Smoking is a risk factor for vocal leukoplakia (OR 4.57, 95% CI 3.0–7.0; $p < 0.01$) and LSCC (OR 5.87, 95% CI 4.1–8.4; $p < 0.01$). Alcohol consumption is also a risk factor for vocal leukoplakia (OR 4.31, 95% CI 2.8–6.6; $p < 0.01$) and LSCC (OR 5.37, 95% CI 3.7–7.7; $p < 0.01$). The classification, clinical stage of LSCC and the lymph node status are detailed in Table 1.

Keratin-8 and Keratin-18 Genotype Frequency in the Controls, Vocal Leukoplakia, and LSCC Patients

Four SNPs (RS1907671, RS2035875, RS2035878, and RS4300473) in keratin-8 gene and 2 SNPs (RS2070876 and RS2638526) in keratin-18 gene were chosen for genotyping. The distributions of keratin-8 and keratin-18 polymorphisms among cases of vocal leukoplakia, LSCC, and the controls are summarized in Tables 2 and 3. Our results showed that the keratin-8 and keratin-18 SNPs were associated with the risk of vocal leukoplakia and LSCC.

The keratin-8 RS1907671 heterozygote AC genotype exhibited significantly increased risk of developing vocal leukoplakia (OR 2.73, 95% CI 1.75–4.26, $p < 0.01$) and LSCC (OR 1.84, 95% CI 1.25–2.54, $p < 0.01$). We also observed a 1.56-fold increased susceptibility for vocal leukoplakia associated with the C allele (OR 1.56, 95% CI 1.17–2.08, $p = 0.002$). In the keratin-8 RS2035875, the CT and TT genotypes exhibited significantly decreased risk of developing LSCC (OR 0.48, 95% CI 0.33–0.71, $p < 0.01$ and OR 0.57, 95% CI 0.38–0.85, $p < 0.01$), and the T allele also showed decreased risk of developing LSCC (OR 0.66, 95% CI 0.52–0.83, $p < 0.01$). For the keratin-8 RS2035878, the homozygote GG genotype and the G allele both exhibited significantly decreased risk of developing vocal leukoplakia (OR 0.42, 95% CI 0.23–0.79, $p = 0.006$ and OR 0.67, 95% CI 0.50–0.89, $p = 0.006$). And in the keratin-8 RS4300473, the heterozygote CG and homozygote GG genotypes demonstrated significantly decreased risk of developing vocal leukoplakia (OR 0.40, 95% CI 0.22–0.72, $p = 0.002$ and OR 0.32, 95% CI 0.17–0.59, $p < 0.01$), and the G allele also showed decreased risk of developing vocal leukoplakia (OR 0.61, 95% CI 0.46–0.82, $p < 0.01$).

For keratin-18 RS2070876, the heterozygote CT genotype illustrated significantly decreased risk of developing LSCC (OR 0.56, 95% CI 0.32–0.96, $p = 0.034$), and in the RS2638526 SNP, the CT and TT genotypes both demonstrated decreased risk of vocal leukoplakia (OR 0.36, 95% CI 0.18–0.72, $p = 0.003$ and OR 0.43, 95% CI 0.21–0.86, $p = 0.016$) and LSCC (OR 0.28, 95% CI 0.16–0.51, $p < 0.01$ and OR 0.40, 95% CI 0.22–0.72, $p = 0.002$).

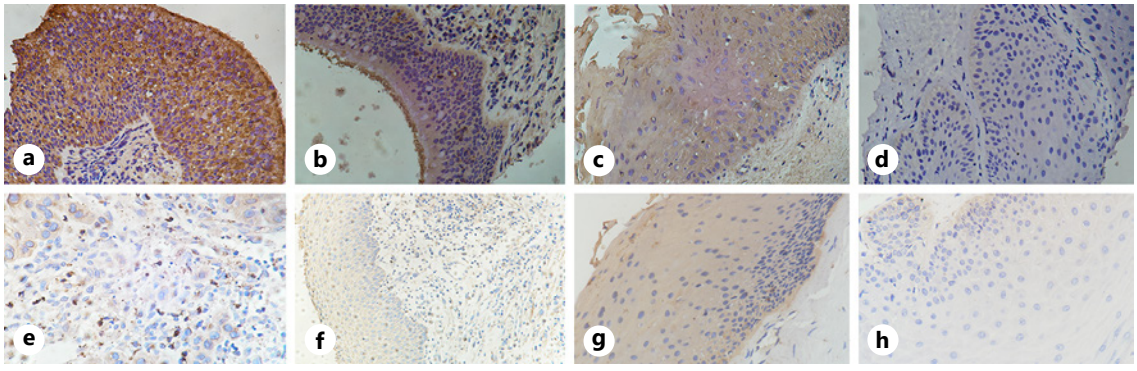
IHC Staining of Keratin-8 and Keratin-18 in Vocal Leukoplakia, LSCC, and Vocal Polyp Patients

As shown in Table 4 and Figure 1, in the IHC staining of keratin-8, a total of 109 cases of vocal leukoplakia, 14 cases of LSCC and 31 cases of vocal polyp were enrolled

Table 4. The IHC results of keratin-8 in vocal leukoplakia, LSCC, and vocal polyp patients

	Vocal leukoplakia				LSCC		Vocal polyp	
	total	low-risk group	high-risk group	*p* value	total	*p* value	total	*p* value
Number	109	64	45		14		31	
IHC grade								
Negative	9	8	1		6		12	
Weakly positive	77	41	36		8		19	
Positive	16	10	6		–		–	
Strongly positive	7	5	2		–		–	
IHC Score, average ± SD	4.30±2.98	4.30±3.25	4.31±2.59	$p1 = 1.000$	1.29±1.25	$p2 = 0.015$	2.06±1.93	$p3 < 0.001$ $p4 = 0.776$

*p*1, *p* value calculated between low-risk group and high-risk group in vocal leukoplakia with SPSS; *p*2, *p* value calculated between LSCC and leukoplakia with SPSS; *p*3, *p* value calculated between vocal polyp and vocal leukoplakia with SPSS; *p*4, *p* value calculated between vocal polyp and LSCC with SPSS.



Color version available online

Fig. 1. Immunostaining of keratin-8 in the vocal fold tissues. Immunostaining of keratin-8 in the vocal fold biopsies from patients with vocal leukoplakia, LSCC (glottic type), and vocal polyp was performed as described in the Methods. Representative images of strongly positive staining (**a**), positive staining (**b**), weakly positive staining (**c**), and negative staining (**d**) in patients with vocal leukoplakia. Representative images of weakly positive staining (**e**) and negative staining (**f**) in patients with LSCC. Representative images of weakly positive staining (**g**) and negative staining (**h**) in patients with vocal polyp.

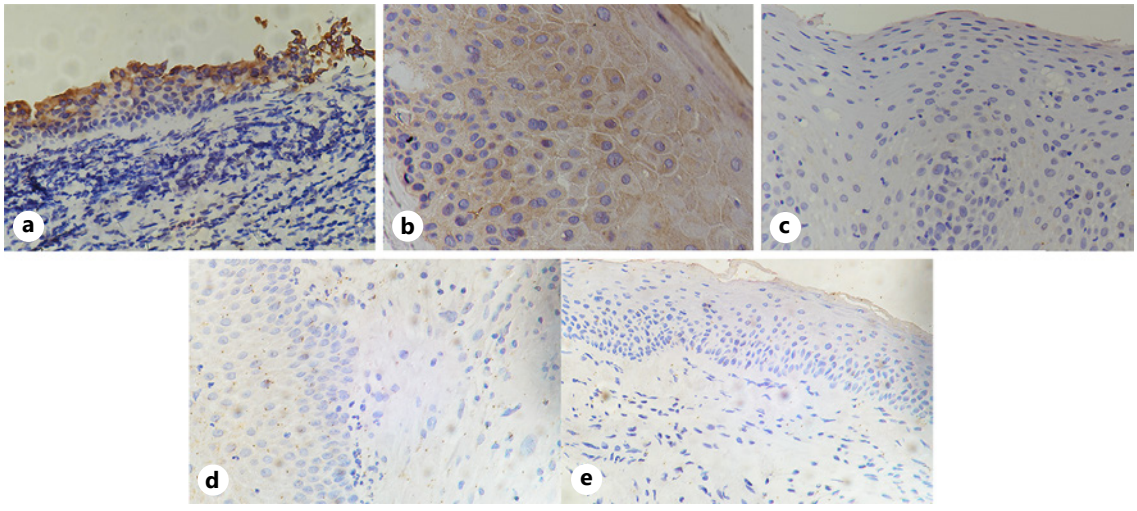
in the study. In the 109 cases of vocal leukoplakia, 9 cases (8.3%) were negative, 77 cases (70.6%) were weakly positive, 16 cases (14.7%) were positive, and 7 cases (6.4%) were strongly positive; the average IHC score of vocal leukoplakia group was 4.30 ± 2.98. In the subgroup analysis of vocal leukoplakia, the average IHC scores of low-risk group and high-risk group were 4.30 ± 3.25 and 4.31 ± 2.60, respectively. No statistical difference was observed between the subgroups ($p = 1.000$). In the LSCC group, 6 cases (42.9%) were negative, and 8 cases (57.1%) were weakly positive for keratin-8 IHC staining. The average IHC score of the LSCC group was 1.29 ± 1.25, which was much lower than that of the vocal leukoplakia group ($p = 0.015$) and vocal polyp group ($p < 0.001$) with statistical differences. In the vocal polyp group, there were 12 cases (38.7%) with negative staining and 19 cases (61.3%) with weakly positive staining, and the IHC score was 2.06 ± 1.93. The comparison between vocal leukoplakia and polyp groups showed no statistical difference ($p = 0.776$).

As shown in Table 5 and Figure 2, in the IHC staining of keratin-18, a total of 96 cases of vocal leukoplakia, 14 cases of LSCC and 19 cases of vocal polyp were enrolled in the study. In the 96 cases of vocal leukoplakia group, 34 cases (35.4%) were negative, 50 cases (52.1%) were weakly positive, and 12 cases (12.5%) were positive for keratin-18 IHC staining; the average IHC score of vocal

Table 5. The IHC results of keratin-18 in vocal leukoplakia, LSCC, and vocal polyp patients

	Vocal leukoplakia				LSCC		Vocal polyp	
	total	low-risk group	high-risk group	*p* value	total	*p* value	total	*p* value
Number	96	36	60		14		19	
IHC grade								
Negative	34	10	24		14		19	
Weakly positive	50	18	32		–		–	
Positive	12	8	4		–		–	
Strongly positive	–	–	–		–		–	
IHC score, average ± SD	2.00±2.23	2.67±2.74	1.60±1.79	$p1 = 0.193$	0.00±0.00	$p2 = 0.022$	0.00±0.00	$p3 < 0.001$ $p4 = 1.000$

*p*1, *p* value calculated between low-risk group and high-risk group in vocal leukoplakia with SPSS; *p*2, *p* value calculated between LSCC and leukoplakia with SPSS; *p*3, *p* value calculated between vocal polyp and vocal leukoplakia with SPSS; *p*4, *p* value calculated between vocal polyp and LSCC with SPSS.



Color version available online

Fig. 2. Immunostaining of keratin-18 in the vocal fold tissues. Immunostaining of keratin-18 in the vocal fold biopsies from patients with vocal leukoplakia, LSCC (glottic type), and vocal polyp was performed as described in the Methods. Representative images of positive staining (**a**), weakly positive staining (**b**), and negative staining (**c**) in patients with vocal leukoplakia. Representative images of negative staining in patients with LSCC (**d**) and vocal polyp (**e**).

leukoplakia group was 2.00 ± 2.23. In the subgroup analysis of vocal leukoplakia, the average IHC scores of high-risk group and low risk group were 2.67 ± 2.74 and 1.60 ± 1.80, respectively. No statistical difference was observed between the subgroups ($p = 0.193$). In the LSCC and vocal polyp groups, all cases were negative for keratin-8 IHC staining. The average IHC scores of the LSCC and vocal polyp group were both much lower than those of the vocal leukoplakia group ($p = 0.022$ and $p < 0.001$).

Discussion

As a common precancerous lesion of the LSCC, vocal leukoplakia is characteristic with keratinization and hyperplasia or different grades of dysplasia pathologically, indicating a trend of malignant progression to carcinoma. Some potential risk factors, such as voice abuse, tobacco and alcohol, laryngopharyngeal reflux, and inflammation have been deemed as triggers for normal mucosa

progress to dysplasia or LSCC [31, 32]. However, the exact nosogenesis of these factors for vocal leukoplakia is still unclear. Thus, the study of the effect of keratins on the occurrence and progression of vocal leukoplakia is clinically needed.

The pathogenesis and cancerization mechanism of vocal leukoplakia is complicated, and as the main pathological feature of vocal leukoplakia is keratinization, many studies focus on the keratins family. In recent years, a number of keratins have been evaluated in oral leukoplakia and carcinoma [33], but the study of keratin effects on vocal leukoplakia is rare. Several studies have reported an aberrant expression of keratin-8 and keratin-18 in the squamous cell carcinomas of the esophagus [34] and oral cavity [33]. Previous reports have shown that an increase in keratin-8 and keratin-18 expression in squamous cell carcinomas as well as adenocarcinomas is associated with the metastatic phenotype [23, 35]. Some reports on tumor samples and cell lines demonstrated that the expression of keratin-8 and keratin-18 was associated with the invasion and poor prognosis [21, 27, 36, 37], so the keratin-8/18 pair may play an important role in the pathogenesis and progression of vocal leukoplakia and LSCC.

In the present study, the results demonstrated that the keratin-8 RS1907671 AC genotype and C allele were associated with an increased risk for vocal leukoplakia and LSCC; in the RS2035875 SNP, the CT and TT genotypes and the T allele showed a decreased risk for LSCC; and in the RS2035878 and RS4300473 SNPs, both the genotypes and G allele demonstrated a decreased risk for vocal leukoplakia. Totally, 3 of the 4 SNPs of keratin-8 were shown to be protective factors of vocal leukoplakia and LSCC. The results of keratin-18 genotypes study demonstrated that the CT genotype of RS2070876 SNP was associated with a decreased risk for LSCC, and in the RS2638526 SNP, both the CT and TT genotypes showed a decreased risk for vocal leukoplakia and LSCC. Thus, both of the keratin-18 SNPs were demonstrated as protective factors of vocal leukoplakia and LSCC. Therefore, our study indicated that keratin-8 and keratin-18 could affect the occurrence and progression of vocal leukoplakia on the gene level.

In the IHC tests, keratin-8 and keratin-18 had similar results. Higher IHC scores of keratin-8 and keratin-18 in the vocal leukoplakia group were detected compared to the LSCC group and vocal polyp group, indicating that the downregulation of keratin-8 and keratin-18 protein expression level facilitates the progression to LSCC. But in the subgroup analysis of vocal leukoplakia, no statistical difference was observed between the low risk group and high risk group, so the relationship of the downregulation of keratin-8 protein expression level and the dysplasia degree of vocal leukoplakia in the progression to LSCC is not sure. It is likely due to the small number of enrolled patients, so further research with a larger number of patients is needed to study the relationship between keratin-8 and keratin-18 protein expression level and different pathological grades in vocal leukoplakia. To sum up, keratin-8 and keratin-18 influence the occurrence and progression of vocal leukoplakia not only on the gene level, but also the protein level, so keratin-8 and keratin-18 could be used as valuable biomarkers for vocal leukoplakia. In the present study, we set the vocal polyp group as control group, the IHC score of which was lower than that of vocal leukoplakia group, but with no statistical difference when compared with the LSCC group. And considering the vocal polyp was not exactly equal to the normal tissues because many inflammatory cells were involved in the polyp tissues, the results of the polyp group need more samples to be verified and further discussed.

The pathogenetic mechanism of keratin-8 and keratin-18 in vocal leukoplakia is unclear, and we speculated that it may be related to the epithelial keratosis. The downregulation of keratin-8 and keratin-18 may break the balance of keratins in epithelia, resulting in keratosis progression in the epithelia, which stimulates the normal epithelial cells to transform to tumor cells. In addition, the imbalance of keratins in epithelia may also compromise the cytoskeleton integrality to facilitate tumor invasion and metastasis. Therefore, although in the present study the relationship of keratin-8 and keratin-18 levels and LSCC metastasis was not studied, the gene and protein level tests of keratin-8 and keratin-18 in LSCC patients with and without metastases is still meaningful. So, further study of tumor invasion and migration was required.

In the present study, our data demonstrated that both cigarette smoking and heavy alcohol consumption were associated with an increased risk for vocal leukoplakia and LSCC, indicating that the environmental factors also play an important role in the pathogenesis of vocal leukoplakia and LSCC. For other contributing factors, such as voice abuse and laryngopharyngeal reflux, considering the quantification of the extent of voice abuse and laryngopharyngeal reflux are still under debate, these factors were not discussed in the present study. Therefore, keratin-8 and keratin-18 as quantifiable indexes, are conveniently applied in the clinic.

There are some limitations to this study. First, the sample size in our study was not large, and some subgroup analyses could not be done well. Second, the LSCC patients enrolled for gene tests were of glottic type, supraglottic type, and subglottic type, although the glottic type was predominant. If we could focus on the glottic carcinoma, the study results might be more targeted. Therefore, we suggest that further studies with more patients should be conducted to further verify our results and speculations.

Conclusion

The present study indicated that keratin-8 and keratin-18 gene polymorphisms and tissue protein levels were associated with the occurrence and progression of vocal leukoplakia.

Statement of Ethics

The study protocol was approved by the Medical Research Council of our hospital (No. KJ2008-01) and informed consent was obtained from all patients and healthy (control) individuals.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

H.W. and P.H. designed the study. Y.Y. and J.Z. completed the study, analyzed the data, and wrote the paper.

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