

Pathological Mechanisms of Laryngeal Papillomatosis Based on Laryngeal Epithelial Characteristics

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Objectives: Human papillomavirus (HPV) infects basal cells of the stratified squamous epithelium through micro epithelial trauma. However, laryngeal papillomatosis commonly appears in any site on the laryngeal mucosa not covered by stratified squamous epithelium. The purpose of this study is to clarify pathological mechanisms of laryngeal papillomatosis based on the characteristics of the laryngeal epithelium.

Study Design: Morphological and immunohistochemical study.

Methods: Larynges from one newborn and two adults were used. Morphological differences in the laryngeal squamo-ciliary junction (ISCJ) were compared to those in the cervical squamo-columnar junction (cSCJ) in a resected cervix uterus. Morphological characteristics of laryngeal epithelial distribution were also compared between the newborn and adult larynges. Immunohistochemical evaluations were performed using p63 (an epithelial stem-cell marker) and integrin- α 6 (a cellular HPV receptor).

Results: Morphological differences were noted between the ISCJ and the cSCJ. The ISCJ was present in the adult, but not the newborn supraglottis. Goblet cells in the pseudostratified ciliated columnar epithelium were also found in the adult but not the newborn larynx. In addition, basal cells of the stratified squamous epithelium as well as the pseudostratified ciliated columnar epithelium expressed p63 and integrin- α 6 in both newborn and adult larynges.

Conclusions: HPV can infect any immature laryngeal epithelium with or without the ISCJ. Squamous metaplasia of pseudostratified ciliated columnar epithelium with a latent HPV infection can also cause tumorigenesis.

Key Words: Laryngeal papilloma, human papillomavirus, squamo-ciliary junction, epithelial stratification.

Level of Evidence: N/A

INTRODUCTION

Laryngeal papillomatosis, a well-known benign neoplasm of the human larynx, is caused by human papillomavirus (HPV) types 6 and 11.^{1,2} Currently, it is widely accepted that the pathogenesis of HPV infection is limited to epithelial stem cells such as basal cells of the stratified epithelium, and is caused by micro epithelial injury.^{3,4} These proliferating cells infected by HPV in episomal forms promote tumorigenesis.

In the cervix uteri, the cervical squamo-columnar junction (cSCJ), which is the transitional region between the stratified squamous and simple columnar epithelium, is the most common site for development of condyloma acuminatum caused by HPV infection. The laryngeal mucosa also has a similar junction: the laryngeal squamo-ciliary junction (ISCJ) between the stratified squamous

and pseudostratified ciliated columnar epithelium. Laryngeal papilloma often develops at sites with ISCJ⁵ and is histologically characterized by a fibrovascular core of connective tissue covered by stratified squamous epithelium. These features are similar to those of condyloma acuminatum.

However, the laryngeal papilloma often appears on any sites of the laryngeal mucosa not covered by genuine stratified epithelium. In particular, juvenile onset laryngeal papilloma caused by vertical transmission of HPV can develop and recur in the supraglottic region without a ISCJ in a newborn larynx.⁶ The pathogenesis of laryngeal papillomatosis is different from that of condyloma acuminatum caused by HPV infection, the latter of which develops at the cSCJ.

The purpose of this study is to clarify the pathological mechanisms of laryngeal papillomatosis based on laryngeal epithelium characteristics.

MATERIALS AND METHODS

Larynges from autopsies of two normal adults and one newborn, as well as two normal adult cervixes uteri from surgical specimens were prepared and fixed in 10% formalin, dehydrated in graded ethanol, and embedded in paraffin. Each specimen was sectioned at 4 μ m thickness and mounted on glass slides.

Morphological Characteristics of Laryngeal and Cervical Uterine Epithelium

After staining each section with hematoxylin-eosin, morphological characteristics of the laryngeal and cervical uterine epithelium

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Conflict of Interest: None

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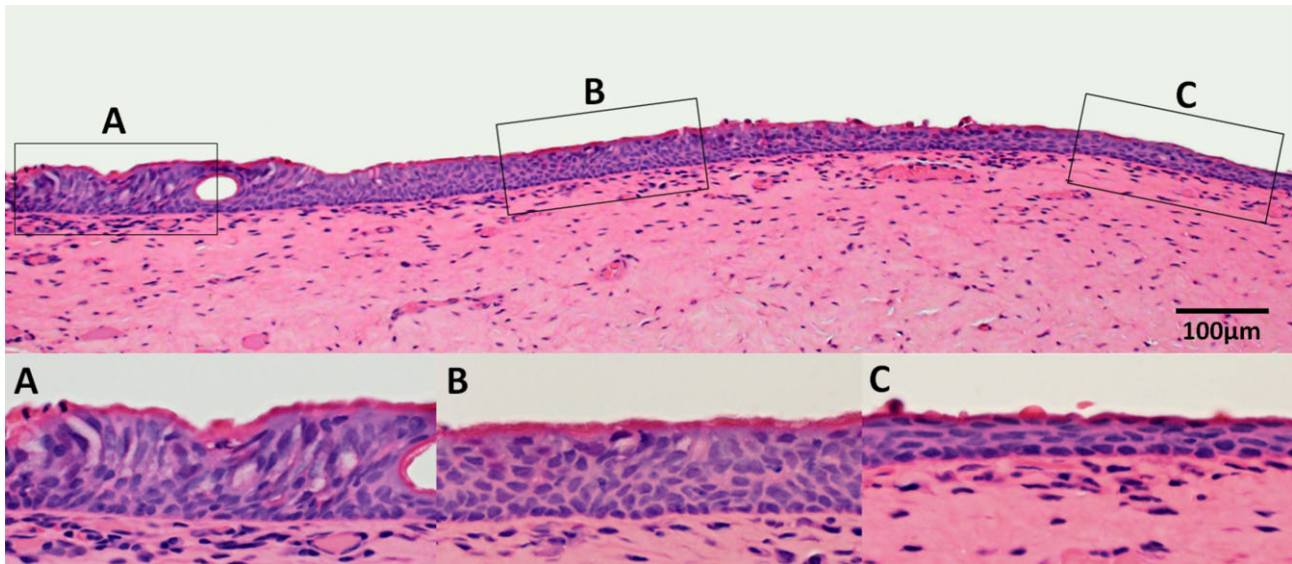


Fig. 1. Histology of the laryngeal squamo-ciliary junction (Hematoxylin-Eosin stain). The laryngeal squamo-ciliary junction consists of the pseudostratified ciliated columnar epithelium (A), the transitional area (B), and stratified squamous epithelium (C). In the stratified squamous epithelium, cell shape is cuboidal in the basal layer, gradually becoming flatter toward the superficial layer. The pseudostratified ciliated columnar epithelium is composed of a single cell layer, many ciliated columnar cells, and some goblet cells with mucus in the cytoplasm. The transitional area between the stratified squamous epithelium and the pseudostratified ciliated columnar epithelium is composed primarily of stratified cuboidal cells. In the transitional area, the ciliated cells and goblet cells are also identified, but fewer of these cells are noted relative to the number of those in the pseudostratified ciliated columnar epithelium. The component cells in the laryngeal squamo-ciliary junction gradually changes from the pseudostratified ciliated columnar epithelium to the stratified squamous epithelium. No obvious boundary between the stratified epithelium and pseudostratified ciliated columnar epithelium is identified in the laryngeal squamo-ciliary junction.

were observed under a light microscope. Differences between the ISCJ and the cSCJ were compared, as was the epithelial distribution between newborn and adult larynges at these subsites.

Immunohistochemical Investigations of Laryngeal Epithelium

Sections of adult larynges were deparaffinized, hydrated, and rinsed with 0.01 mol/L phosphate-buffered saline (PBS) at pH 7.4. Antigen retrieval by microwave irradiation was conducted with Target Retrieval Solution, pH 9 (S2367, Dako, Glostrup, Denmark) for 20 minutes. Sections were blocked with 3% hydrogen peroxide for 10 minutes, then incubated with the following primary antibodies for 90 minutes at room temperature: 1:300 diluted anti-p63 (BC4A4) (ab735, Abcam, Cambridge, UK), and 1:300 diluted anti-integrin alpha 6 (EPR5578) (ab124924, Abcam, Cambridge, UK). After rinsing with PBS and incubating with universal secondary antibodies conjugated with horseradish peroxidase-labeled amino acid polymer (Envision/HRP, Dako, Glostrup, Denmark) at room temperature for 30 minutes, the immunoreactive proteins were visualized with 3,3'-diaminobenzidine tetrahydrochloride for 10 minutes. Each section was counterstained with hematoxylin, dehydrated with xylene, and mounted.

RESULTS

Morphological Differences Between Laryngeal SCJ and Cervix Uterine SCJ

The ISCJ consisted of the stratified squamous epithelium, pseudostratified ciliated columnar epithelium, and the transitional area (Fig. 1). In the stratified squamous epithelium, cell shape was cuboidal in the basal layer, gradually becoming flatter toward the superficial

layer. The pseudostratified ciliated columnar epithelium was composed of a single cell layer, many ciliated columnar cells, and some goblet cells with mucus in the cytoplasm. The transitional area between the stratified squamous epithelium and the pseudostratified ciliated columnar epithelium was composed primarily of stratified cuboidal cells. In the transitional area, the ciliated cells and goblet cells were also identified, but fewer of these cells were noted relative to the number of those in the pseudostratified ciliated columnar epithelium. Thus, the component cells in the ISCJ gradually changed from the pseudostratified ciliated columnar epithelium to the stratified squamous epithelium. No obvious boundary between the stratified epithelium and pseudostratified ciliated columnar epithelium was identified in the ISCJ.

The cSCJ consisted of the stratified squamous epithelium and simple columnar epithelium (Fig. 2). The simple columnar epithelial cells had clear mucus-rich cytoplasm, and the cell nuclei formed close to the basal lamina. In the stratified squamous epithelium, cell shape was cuboidal in the basal layer, gradually becoming flatter toward the superficial layer. Stratified squamous epithelial cells had eosinophilic cytoplasm and no mucus. The cSCJ had no transitional area, and the boundary between the stratified squamous epithelium and the single columnar epithelium was clearly defined.

Distribution and Morphological Characteristics of the Human Adult and Newborn Laryngeal Epithelium

In adult larynges, the stratified squamous epithelium was distributed in the upper half of the epiglottis and

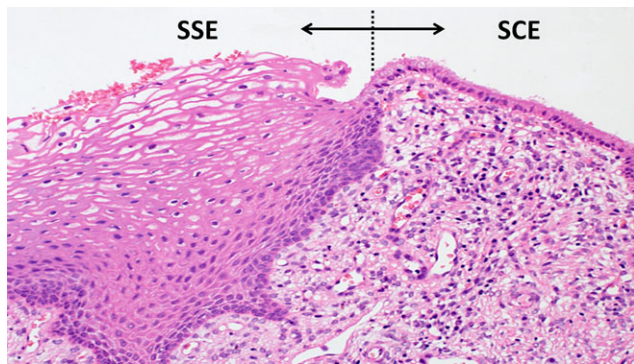


Fig. 2. Histology of the cervical squamo-columnar junction (Hematoxylin-Eosin stain). SSE: stratified squamous epithelium; SCE: simple columnar epithelium. The cervical squamo-columnar junction consists of the stratified squamous epithelium and simple columnar epithelium. The simple columnar epithelial cells have clear mucus-rich cytoplasm, and the cell nuclei formed close to the basal lamina. In the stratified squamous epithelium, cell shape is cuboidal in the basal layer, gradually becoming flatter toward the superficial layer. Stratified squamous epithelial cells have eosinophilic cytoplasm and no mucus. The cervical squamo-columnar junction has no transitional area, and the boundary between the stratified squamous epithelium and the single columnar epithelium is clearly defined.

vocal folds (Fig. 3A, I). The epithelium consisted of approximately five cell layers. In contrast, the pseudostratified columnar epithelium was distributed to the lower half of the epiglottis, vestibular folds (false vocal folds), laryngeal ventricles, and the subglottic region (Fig. 3C, E, G, K). The pseudostratified columnar epithelium had goblet cells.

In the newborn larynx, the stratified squamous epithelium was only distributed to the vocal folds (Fig. 3J). Vocal fold epithelium consisted of approximately three cell layers. Pseudostratified columnar epithelium was distributed to the entire epiglottis, vestibular folds (false vocal folds), laryngeal ventricles, and the subglottic region (Fig. 3B, D, F, H, L). The pseudostratified columnar epithelium had few goblet cells.

Immunohistochemical Investigations of Laryngeal Epithelium

Basal cells in the stratified squamous epithelium expressed p63 in the nucleus (Fig. 4A). In the pseudostratified ciliated columnar epithelium, cells in the basal layer also expressed p63 (Fig. 4B).

Cells in the lower half of the stratified squamous epithelium expressed integrin- $\alpha 6$ in the cytoplasm (Fig. 4C). All pseudostratified ciliated columnar epithelial cells also expressed integrin- $\alpha 6$ (Fig. 4D). These immunohistochemical findings were identified consistently in all larynges.

DISCUSSION

This study revealed several novel findings. First, similar to that of the cSCJ, the morphology of the lSCJ creates a favorable environment for HPV infection. Second, the newborn laryngeal epithelium is susceptible to HPV infection. Third, HPV is capable of infecting not only stratified squamous epithelium, but also pseudostratified ciliated columnar epithelium.

Characteristics of the Laryngeal SCJ for HPV Infection

The present study found that the transitional area of the lSCJ had poorly differentiated epithelial cells with characteristics of both stratified squamous cells and pseudostratified ciliated columnar epithelial cells. A previous study by Tucker et al.⁷ used a scanning and transmission electron microscopy to demonstrate that the transitional area of the lSCJ has a gradual transition, rather than a sharp demarcation of cellular structure without cilia. The “stratified columnar epithelial cells” of the transitional area can ultimately evolve into a genuine stratified squamous cellular structure. On the other hand, the cSCJ with no transitional area showed an obvious boundary between the stratified squamous epithelium and single columnar epithelium. In addition, compared to the stratified squamous epithelium, the single columnar epithelium of the cSCJ is more vulnerable to external stimuli.⁸ Therefore, mucosal erosion and inflammation occur more easily in the cSCJ. Actually, squamous metaplasia as an adaptive reaction to the external stimuli frequently occurs in the simple columnar epithelium of the cSCJ.^{9,10} One similarity between the two SCJs is that both have a junction consisting of monolayered and stratified epithelium. Condyloma acuminatum and laryngeal papilloma frequently develop in each SCJ.⁵ It is known that the pathogenesis and histopathology of laryngeal papilloma resemble those of condyloma acuminatum. HPV is highly tissue-specific and characterized by a specific mode of interaction with the epithelia it infects.¹¹ Phylogenetic assemblages coincide with biological and pathological properties.¹² Papilloma development may be associated with immaturity of cells in the transitional area of the lSCJ.

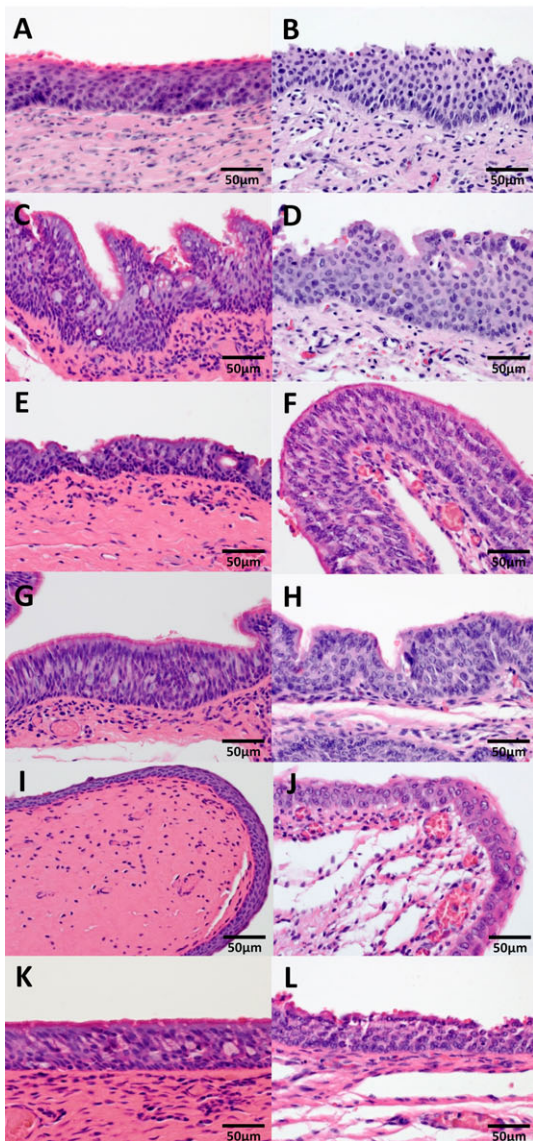
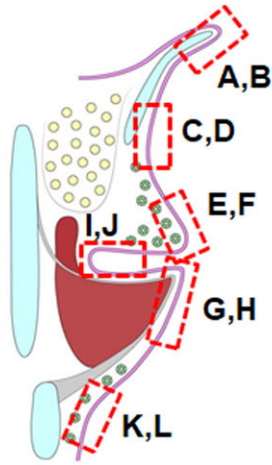
Characteristics of Newborn Laryngeal Epithelium for HPV Infection

In the newborn larynx, we observed less epithelial stratification in the vocal folds and fewer goblet cells in the pseudostratified ciliated columnar epithelium relative to those in the adult larynges, indicating the immaturity of the newborn laryngeal epithelium. HPV can reach basal cells of the stratified squamous epithelium more easily in newborn vocal folds than those of adults. Previously, Hopp et al. revealed that the newborn epithelium of the larynx is embryonic and polyhedral. They also found that the embryonic epithelium of the vocal folds becomes cuboidal and then stratified and squamous in shape.⁶ Most of the newborn laryngeal mucosa is composed of columnar or pseudostratified ciliated epithelium. These epithelial immaturities of the newborn larynx can allow HPV to infect any sites of the laryngeal mucosa without genuine stratified epithelium.

Mechanisms of Papilloma Development in HPV-Infected Epithelium

HPV is a nonenveloped virus that contains a double-stranded, closed circular DNA genome associated with histone-like proteins and protected by capsid proteins.

The viral capsid binds initially to the basal cell layer through microlesions, and infection occurs when activated stratified squamous epithelial cells move to the upper



layers of the epithelium. HPV genomes replicate in the nuclei of cells in the basal layer, where viral replication is considered nonproductive and the virus establishes itself as a low-copy-number episome by using the host DNA replication machinery. In this way, viral proteins are expressed at very low levels in undifferentiated cells, which contributes to immune evasion and viral persistence. However, when HPV-infected cells leave the basal layer, they undergo differentiation, and high levels of viral protein synthesis are induced.¹⁰

Our immunohistochemical study indicated that HPV is able to infect not only stratified squamous epithelium but also pseudostratified ciliated columnar epithelium because both types of epithelium express p63 and integrin- $\alpha 6$. p63 is a nuclear protein present in basal cells of normal stratified squamous and other epithelium.¹³⁻¹⁵ This protein plays a crucial role in the regulation of stem cell commitment in the epithelium.^{13,16,17} In HPV infected epithelial cells, the expression of p63 is also necessary for regulating both differentiation-dependent viral genome amplification and late gene expression.¹⁸ Integrin- $\alpha 6$, a cell adhesion molecule, is one of the candidates for the HPV receptors.¹⁹ An immunohistochemical positive for integrin- $\alpha 6$ indicates that both kinds of epithelium have cells with a receptor for HPV. Thus, epithelial cells that express both p63 and integrin- $\alpha 6$ can be infected with HPV.

Steinberg et al. reported on DNA sequencing studies of biopsy samples from patients with laryngeal papilloma during clinical remission. They describe that HPV type 6 was detected in biopsy samples from the epiglottis and the false vocal fold in an asymptomatic young boy, who developed active papilloma of the true vocal fold as an adult. Their report is consistent with our finding that HPV can infect the pseudostratified ciliated columnar epithelium in the epiglottis or false vocal fold. However, HPV infection alone did not lead to papilloma development in the monolayered epithelium.

HPV-DNA replication and the tumorigenic transformation increase together with the differentiation of stratified squamous epithelial cells.¹⁰ Normal stratified squamous epithelial cells differentiate from the basal layer to the superficial layer. As epithelial stem cells, basal cells undergo mitosis to provide for cell renewal and are pushed toward the surface by new cells in the basal layer.²⁰ When HPV-infected basal cells differentiate into

Fig. 3. Comparison of the histology and distributions of laryngeal epithelium between adult (A, C, E, G, I, K) and newborn (B, D, F, H, J, L) larynges. Upper part of epiglottis (A, B). Lower part of epiglottis (C, D). Vestibular folds (E, F). Ventricle (G, H). Vocal fold (I, J). Subglottis (K, L). In adult larynges, the stratified squamous epithelium is distributed in the upper half of the epiglottis (A) and vocal folds (I). The epithelium consisted of approximately five cell layers. The pseudostratified columnar epithelium is distributed to the other regions (C, E, G, K). The pseudostratified columnar epithelium has goblet cells. In the newborn larynx, the stratified squamous epithelium is only distributed to the vocal folds (J). Vocal fold epithelium consists of approximately three cell layers. Pseudostratified columnar epithelium is distributed to the other regions (B, D, F, H, L). The pseudostratified columnar epithelium has few goblet cells.

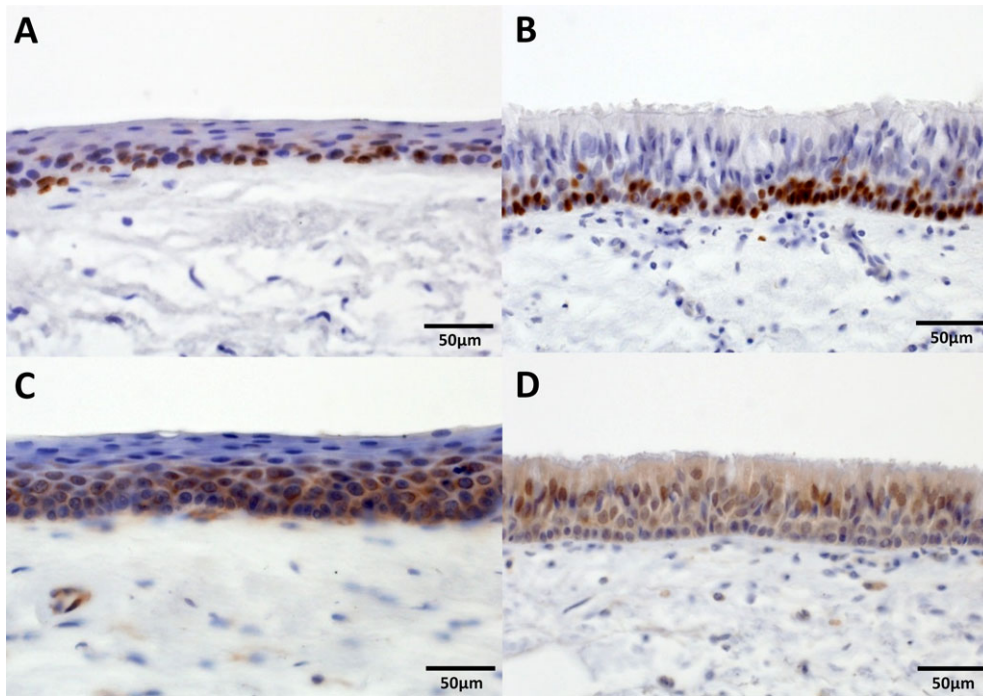


Fig. 4. Immunohistochemistry of the laryngeal stratified squamous epithelium (A, C) and pseudostratified ciliated columnar epithelium (B, D) in an adult larynx. Basal cells in the stratified squamous epithelium express p63 in the nucleus (A). In the pseudostratified ciliated columnar epithelium, cells in the basal layer also express p63 (B). Cells in the lower half of the stratified squamous epithelium express integrin- α 6 in the cytoplasm (C). All pseudostratified ciliated columnar epithelial cells also express integrin- α 6 (D).

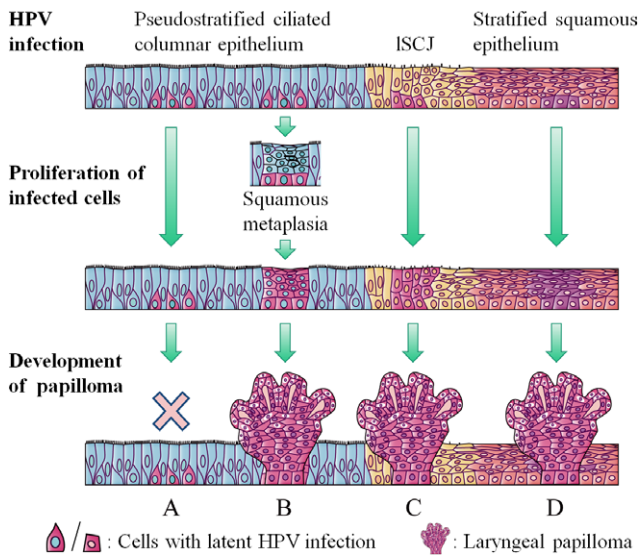


Fig. 5. Schema of the developmental mechanism of laryngeal papillomatosis. In the laryngeal squamo-ciliary junction as well as the stratified squamous epithelium, HPV-DNA replication can cause tumorigenic transformation along with epithelial stratification (C, D). On the other hand, even when HPV infects the pseudostratified ciliated columnar epithelium, tumorigenic transformation does not occur without epithelial stratification (A). However, once squamous metaplasia occurs in pseudostratified ciliated columnar epithelium with a latent HPV infection, this monolayered epithelium undergoes stratification with high levels of viral protein. As a result, laryngeal papilloma can develop in mucosal regions covered by pseudostratified ciliated columnar epithelium (B). HPV infection in epithelial stem cells but also the stratification of the HPV-infected epithelial stem cells is necessary for laryngeal papilloma development. ISCJ = laryngeal squamo-ciliary junction.

superficial squamous cells, HPV-DNA replication is also induced with high levels of viral protein synthesis.¹⁰

Therefore, in the ISCJ as well as the stratified squamous epithelium, HPV-DNA replication can cause tumorigenic transformation along with epithelial stratification (Fig. 5C,D). On the other hand, even when HPV infects the pseudostratified ciliated columnar epithelium, tumorigenic transformation does not occur without epithelial stratification (Fig. 5A). However, once squamous metaplasia occurs in pseudostratified ciliated columnar epithelium with a latent HPV infection, this monolayered epithelium undergoes stratification with high levels of viral protein. As a result, laryngeal papilloma can develop in mucosal regions covered by pseudostratified ciliated columnar epithelium (Fig. 5B). The present study indicates that not only HPV infection in epithelial stem cells but also the stratification of the HPV-infected epithelial stem cells is necessary for laryngeal papilloma development.

In cases of juvenile onset laryngeal papilloma, this may likely explain why papilloma develops in multiple regions or recurs in the supraglottic region. The newborn larynx has no ISCJ and no squamous metaplasia by nature. However, squamous metaplasia can occur after birth when the laryngeal epithelium is exposed to external stimuli such as pathogenic organisms or polluted air. The immature laryngeal epithelium of newborns can also be easily infected with HPV, resulting in multiple development and recurrence.

A limitation of this study is that a small number of laryngeal specimens were used because it is difficult to harvest a large number of normal human larynges, especially newborn larynges. In addition, although laryngeal

papilloma is a HPV infection dependent disease, normal laryngeal epithelium were examined histologically and immunohistochemically in this study. The exact mechanism of development of papilloma from HPV infected epithelium is still not clear and, additionally, its correlation to the activities of HPV genome replication in both stratified squamous and pseudostratified ciliated columnar epithelium is also not clear. Further research in vitro or vivo into HPV infected epithelium will allow us to clarify the mechanism of papilloma development.

CONCLUSION

HPV can infect not only the stratified squamous epithelium but also the pseudostratified ciliated columnar epithelium in the larynx. Additionally, immature laryngeal epithelium such as the transitional area in the LSCJ and the newborn laryngeal epithelium can be susceptible to HPV.

Papilloma development requires epithelial stratification as well as HPV infection in order to proceed. Besides the morphological characteristics and immaturity of the newborn laryngeal epithelium, squamous metaplasia after birth in pseudostratified ciliated columnar epithelium with latent HPV infection can also cause tumorigenesis.

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