

Morphological Alteration of the Salivary Duct by Chronic Inflammation with Histopathological,

Ultrastructural and Immunohistochemical Study

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## **Abstract**

To clarify the process to squamous metaplasia, we investigated ultrastructural features of the salivary ducts. And immunohistochemical staining was performed to determine the expression of cytoskeletal and intercellular adhesion molecules associated with morphological alterations of the salivary ducts. This study comprised 117 patients with chronic sialadenitis. The salivary ducts of the patients were microscopically classified into the following three groups: dilated ducts; hyperplastic ducts; and squamous metaplastic ducts. Squamous metaplastic ducts occurred in 34 of the 117 patients (29.0%). Immunohistochemical staining was performed in 25 patients with sialadenitis in whom the histological specimens were not decalcified at the time of preparation. Histopathological and ultrastructural analyses of the hyperplastic ducts showed hyperplasia of cuboidal basal cell-like cells with relatively high nucleus/cytoplasmic ratio. Immunohistochemically, hyperplastic basal cell-like cells were positive for p63, CK34 $\beta$ E12, CK5/6, CK19, and E-cadherin, thus reflecting basal cell characteristics. In immature squamous metaplastic ducts, positive reaction for CK18, CK5/6 and E-cadherin were attenuated. In mature

squamous metaplastic ducts, CK13 appeared instead of E-cadherin and  $\beta$ -catenin. This study showed that dilation of ducts was due to abnormal physical irritation and that chronic irritation causes basal cell hyperplasia as a biological adaptive response. Basal cell hyperplasia and squamous epithelium characteristics were also demonstrated by ultrastructural analysis by using electron microscopy. These changes are also supported by the fact that cytokeratin in ductal epithelial cells undergo transition from a glandular epithelial type to mucosal type. These results suggest that basal cell hyperplasia is a squamous metaplastic process.

## Introduction

Salivary gland ducts are histologically surrounded by columnar epithelium, and they discharge secretions to mucosal epithelial surfaces. Sialadenitis is a condition caused by bacterial infection, viral infection, autoimmune disease, or as a complication of radiotherapy (1).

With a transition to chronic sialadenitis, histopathological changes of the ducts include dilation and hyperplastic or squamous metaplasia. Ductal dilation may be caused by exocrine dysfunction due to chronic irritation or inflammatory changes. In addition, when abnormal stimulation or inflammation continues or increases, stratification and squamous metaplasia of the ducts may occur as a biological adaptive response.

Metaplasia has been referred to differentiation of terminally differentiated cells into another type of cell due to the effects of long-term abnormal stimulation in the past (2). Recently, metaplasia occurs not because of transdifferentiation of already differentiated cells, but rather because of reprogramming of stem cells to new types of differentiation (3). Metaplasia occurs in almost all tissues and organs (4, 5), typically with changes from columnar epithelium to

stratified squamous metaplasia. Some immunohistochemical studies have investigated stratified squamous metaplasia of tissues covered by columnar epithelium, including the breast (6), prostate gland (7), bronchus (8), and uterine cervix (9); however, the sequential changes from regular ductal tissue to squamous metaplasia have seldom been reported (9). Moreover, comparisons of the ultrastructure of regular ducts and squamous metaplastic ducts by using electron microscopy have not been reported. The salivary ducts are also a frequent site of squamous metaplasia (2). Squamous metaplasias in salivary gland tumors and necrotizing sialometaplasia have been reported (10-12), but reports about squamous metaplasia of the regular salivary ducts have been rare (13).

Therefore, to observe the process to squamous metaplasia, we investigated ultrastructural features of morphological alterations of the salivary ducts. In addition, we performed immunohistochemical staining to determine the expression of cytoskeletal and intercellular adhesion molecules associated with morphological alterations of the salivary ducts.

## Materials and Methods

### *1) Clinicopathological and histopathological evaluations*

This study comprised 117 patients with chronic sialadenitis based on histological diagnosis of surgically resected specimens between 1997 and 2011 at Nihon University Hospital at Matsudo. Sixty-two patients had chronic sialadenitis alone, and 55 had sialolithiasis and chronic sialadenitis. Table 1 summarizes the clinico- and histopathological findings.

The ductal epithelium was reexamined by microscopy with hematoxylin and eosin (HE)-stained specimens from each patient. The findings were classified into the following four groups; dilated ducts (bilayer without metaplasia), hyperplastic ducts (without squamous metaplasia), immature squamous metaplastic ducts, and mature squamous metaplastic ducts.

### *2) Transmission electron microscopy (TEM)*

Parts of the specimen fixed with 10% neutral formalin were washed and refixed with 2% glutaraldehyde and 1% osmium tetroxide. The tissue was then embedded in epoxy resin (Quetol

812, Nisshin EM, Tokyo, Japan) by the usual method. Ultrathin sections were poststained with uranyl acetate and lead citrate, and observed by TEM(JEM-1200 EX II, JEOL, Tokyo, Japan).

### *3) Immunohistochemical staining*

IHC staining was performed in 25 patients with sialadenitis in whom the histological specimens were not decalcified at the time of preparation. In addition, morphologically regular salivary gland duct within these specimens that was distal to the lesions was used as the controls.

Table 2 summarizes these patients.

The specimens used were 4- $\mu$ m thin-tissue sections from paraffin blocks that were prepared at the time of histopathological examination after surgery and stored by the Department of Diagnostic Pathology at the Hospital.

The specimens were prepared by standard techniques. After deparaffinization in a xylene-alcohol series, the specimens were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. The primary antibodies used to analyze the cytoskeleton were



polyclonal rabbit anti-cytokeratin, wide spectrum screening (dilution 1:500, Dako Cytomation, Glostrup, Denmark; pankeratin), anti-cytokeratin 18 antibody (DC10, dilution 1:50, Dako Cytomation; CK18), anti-cytokeratin 19 antibody (RCK108, dilution 1:50, Dako Cytomation; CK19), anti-cytokeratin 5/6 antibody (D5/6 B4, dilution 1:50, Dako Cytomation; CK5/6), anti-cytokeratin 13 antibody (DE-K13, dilution 1:50, Dako Cytomation; CK13), anti-cytokeratin, high molecular weight (34 $\beta$ E12, dilution 1:100, Dako Cytomation; CK34 $\beta$ E12), polyclonal rabbit anti-S100 antibody (dilution 1:500, Dako Cytomation; cytokeratin), and anti-mitochondria antibody (an organelle marker; 113-1, dilution 1:50, Chemicon International, CA; MIT).

The primary antibodies used to analyze cell adhesion were anti-E-cadherin antibody (NCH-38, dilution 1:100, Dako Cytomation; E-cadherin) and anti-beta-catenin antibody ( $\beta$ -catenin-1, dilution 1:200, Dako Cytomation;  $\beta$ -catenin). The primary antibody to analyze the character of epithelial stem cells was anti-p63 protein antibody (4A4, Dilution 1:50, Dako Cytomation; p63).

For antigen activation, pankeratin, CK19, and CK34 $\beta$ E12 were treated with proteinase K (Dako Cytomation) at room temperature for 5 min. CK5/6, E-cadherin,  $\beta$ -catenin, and p63 were treated by using 10 mmol/L Tris buffer (pH 9.0) containing 1 mmol/L EDTA at 111°C in a pressure cooker at 1.54 atm for 10 min. CK18 and CK13 were treated by using 10 mmol/L citrate buffer solution (pH 6.0) in a microwave for 15 min. After antigen activation, each primary antibody was allowed to react at room temperature for 1 hour.

The secondary antibody was ChemMATE Envision (Dako Cytomation). The chromogenic substrate was liquid DAB+ (Dako Cytomation). Counterstaining was performed by using Mayer's hematoxylin. After dehydration and clearing in a xylene-alcohol series, and mounting were performed. In addition, as positive controls for each antigen, regular mucosal tissue and regular salivary gland tissue were used for pankeratin, CK18, CK19, CK5/6, CK13, CK34 $\beta$ E12, S100, E-cadherin,  $\beta$ -catenin and p63. Warthin tumor was used as a positive control for MIT. As negative controls, instead of the primary antibodies, IgG1 negative control (Dako Cytomation) was used for CK18, CK19, CK5/6, CK34 $\beta$ E12, E-cadherin,  $\beta$ -Catenin, and MIT; IgG2a

negative control (Dako Cytomation) was used for CK13 and p63; and immunoglobulin fraction (Dako Cytomation) was used as a negative control for pankeratin and S100.

This study was conducted after obtaining patient consent, and attention was paid to maintaining patient privacy (Ethics Committee Approval No. EC13-001).

#### *4) Statistical analysis*

Statistical analysis, including the Kruskal-Wallis test and Chi-square test, was performed by using SPSS 11.0J software. The level of statistical significance was set as less than 5%.

## **Results**

### *1. Clinicopathological and histopathological findings*

Table 1 summarizes the clinico- and histopathological findings. The 117 patients with chronic sialadenitis (42 men, 75 women; mean age, 56.0 years) comprised significantly more women than men ( $p < 0.001$ ). In addition, 55 of these patients had sialolithiasis and chronic

sialadenitis. This accounted for 47.0% of all patients. There were 26 men and 29 women, with no significant difference in the male: female ratio.

The morphology of the salivary ducts was histopathologically classified into the following three groups: dilated ducts in which a bilayer structure was preserved; hyperplastic ducts with a superficial layer covered by columnar cells and stratified basal cell-like cells; and squamous metaplastic ducts, in which the duct walls were replaced by squamous epithelial-like cells. The squamous metaplastic ducts were further classified into immature squamous metaplasia ducts, in which there was a mixture of cuboidal epithelium and squamous epithelium; and mature squamous metaplastic ducts, which except for the basal and parabasal layers, had a mucosal epithelial structure replaced by squamous epithelial-like cells. Furthermore, the squamous metaplastic ducts often had mixed areas of basal cell-like cells hyperplasia and squamous metaplasia within the same ducts. The squamous metaplastic ducts tended to have sloughing of the superficial epithelium.

Hyperplastic ducts of the ductal epithelium occurred in 22 of the 117 patients (18.8%) with

chronic sialadenitis. This included 6 men and 16 women, and the most common site (11 patients) was the submandibular gland. Squamous metaplasia of the ducts occurred in 34 of the 117 patients (29.0%). This included 21 men and 13 women, and the most common site (26 patients) was also the submandibular gland.

Among the 55 patients with both sialolithiasis and chronic sialadenitis, hyperplasia of the ductal epithelium occurred in 15 patients (27.3%). This included 5 men and 10 women (mean age, 54.6 years), and the most common site (10 patients) was the submandibular gland. Squamous metaplasia of the ducts occurred in 31 of the 55 patients (56.4%). This included 19 men and 12 women (mean age, 56.5 years), and the most common site (25 patients) was also the submandibular gland. No significant differences were found in mean age, male-female ratio, or characteristics of the ductal epithelium based on the presence or absence of sialolithiasis.

## *2. Transmission electron microscopy (Fig. 1)*

Microvilli-line (MV) lumen (L) of an intercalated duct is lined by cuboidal ductal cells (DC), and a myoepithelial cell (MEC) subjacent to the basal lamina (BL). The stratified (hyperplastic) duct (H) consisted of small cuboidal cells (C) with high nuclear/cytoplasmic ratio and mitochondria. The intermediate filament of the cells was intermingled with the dense and mesh-like rough part. The region of squamous metaplasia (S) is composed of flattened cells with centrally oriented nuclear. These cells contain glycogen granules (Gly) and tonofilament bundles (TF), and are joined by well-formed desmosomes (D).

### *3. Immunohistochemical findings (IHC)*

Figure 1 shows the histopathological, ultrastructural staining results, and Figure 2 indicates IHC staining results. Figure 3 shows the results in graphical form.

#### *1) Pankeratin*

IHC staining was positive for pankeratin in all epithelial cells (ductal epithelial cells, basal cells, hyperplastic cells, squamous metaplastic cells, and superficial cells) of the cases.

## 2) *CK5/6*

IHC staining for CK5/6 showed strong positivity in the basal cells of regular ducts and dilated ducts. In hyperplastic ducts, there was strong positivity in basal cells and hyperplastic cells, and moderate positivity in superficial cells. In immature squamous metaplastic ducts, moderate positivity was observed in the basal cells and hyperplastic cells, and weak positivity was seen in the squamous metaplastic cells and superficial cells. The mature squamous metaplastic ducts were negative for CK5/6, except for some minimal expression in the superficial cells.

## 3) *CK13*

IHC staining for CK13 was negative in regular ducts and dilated ducts. In hyperplastic ducts, weakly to moderately positive reaction was found. In immature squamous metaplastic ducts, some hyperplastic cells were positive, and strong positivity was observed in squamous

metaplastic cells and superficial cells. In mature squamous metaplastic ducts, the basal cells were negative for CK13, but diffuse strong positivity was evident in hyperplastic cells, squamous metaplastic cells, and superficial cells.

#### 4) *CK18*

IHC staining for CK18 was strongly positive in the ductal epithelial cells of regular ducts and dilated ducts. In hyperplastic ducts and immature squamous metaplastic ducts, there was moderate positivity in columnar-like superficial cells, and some weak positivity in hyperplastic cells and squamous metaplastic cells. In basal cells of hyperplastic ducts and immature squamous metaplastic ducts, and in mature squamous metaplastic ducts, CK18 was negative.

#### 5) *CK19*

IHC staining for CK19 was moderately positive in the ductal epithelial cells of regular ducts and dilated ducts. In hyperplastic ducts, weak positivity was observed in basal cells, moderate



positivity in hyperplastic cells, and strong positivity in columnar-like superficial cells. In immature squamous metaplastic ducts, weak positivity was seen in basal cells and moderate positivity was seen in hyperplastic cells, squamous metaplastic cells, and columnar-like superficial cells. In mature squamous metaplastic ducts, basal cells were moderately positive, hyperplastic cells were minimally positive, and squamous metaplastic cells and superficial cells were negative for CK19.

#### 6) *CK34βE12*

IHC staining for CK34βE12 was strongly positive in the ductal epithelial cells and basal cells of regular ducts and dilated ducts. In hyperplastic ducts, strongly positive cells were evident in all layers. In immature squamous metaplastic ducts, strong positivity was observed in basal cells and hyperplastic cells, and moderate positivity was seen in squamous metaplastic cells and superficial cells. In mature squamous metaplastic ducts, the basal cells were moderately positive, and the superficial cells were weakly positive for CK34βE12.

### 7) *E-cadherin*

IHC staining for E-cadherin was strongly positive in the basal cells of regular ducts and dilated ducts, and mildly positive in the ductal epithelial cells. In hyperplastic ducts, there was strong positivity in basal cells, and diffuse strong positivity in hyperplastic cells and superficial cells. In immature squamous metaplastic ducts, basal cells were negative, some hyperplastic cells were weakly positive, squamous metaplastic cells were weakly positive, and superficial cells were strongly positive. In mature squamous metaplastic ducts, E-cadherin was minimally expressed in superficial cells.

### 8) *β-catenin*

IHC staining for β-catenin was strongly positive in the basal cells of regular ducts and dilated ducts. In hyperplastic ducts, there was strong positivity in all layers. In immature squamous metaplastic ducts, there was mild positivity in basal cells, moderate positivity in

hyperplastic cells and squamous metaplastic cells, and strong positivity in superficial cells. In mature squamous metaplastic ducts,  $\beta$ -catenin was minimally expressed in squamous metaplastic cells.

#### 9) p63

IHC staining for p63 was positive in the basal cells of regular ducts and dilated ducts. In hyperplastic ducts, there was strong positivity in almost all basal cells, and strong positivity in hyperplastic cells. In immature squamous metaplastic ducts, there was strong positivity in all basal cells, strong positivity in hyperplastic cells, and some positivity in squamous metaplastic cells. In mature squamous metaplastic ducts, basal cells were moderately positive.

#### 10) MIT

IHC staining for MIT showed strong positivity in all layers in regular ducts, dilated ducts, and hyperplastic ducts. In immature squamous metaplastic ducts, there was moderate positivity

in basal cells, hyperplastic cells, and superficial cells; and MIT was minimally expressed in squamous metaplastic cells. In mature squamous metaplastic ducts, basal cells were weakly positive; and hyperplastic cells, squamous metaplastic cells, and superficial cells were slightly positive for MIT.

#### *11) S100*

IHC staining for S100 showed scattered positivity in myoepithelial cells of the intercalated ducts. However, all cells in the dilated ducts, hyperplastic ducts, and squamous metaplastic ducts were negative for S100.

## **Discussion**

Salivary gland ducts are histologically surrounded by columnar epithelium, and they discharge secretions to mucosal epithelial surfaces. Histological examination of the ducts in sialadenitis may show dilation, hyperplasia, and squamous metaplasia. Dilation of the ducts

occurs due to exocrine dysfunction due to chronic irritation and inflammatory changes.

Moreover, if this irritation and inflammation continues or increases, hyperplasia and squamous

metaplasia of the ducts occur as a biological adaptive response. Metaplasia refers to

differentiation of terminally differentiated cells into another type of cell due to the effects of

long-term abnormal stimulation. Metaplasia has previously been reported only to occur in cells

with proliferative capacity and be mainly associated with degeneration and tumorigenesis (2).

Metaplasia occurs in almost all tissues and organs (4, 5), and in particular, columnar epithelium

is more susceptible to mechanical irritation than stratified squamous epithelium. Therefore,

hyperplasia and squamous metaplasia occur in areas covered by columnar epithelium, including

mammary ducts in the breast (6), basal cells in prostate gland ducts (7), ciliated columnar

epithelium in the bronchi (8), and cervical gland epithelium in the uterine cervix (9).

Squamous metaplasia is also frequently observed in the salivary ducts (2). Squamous

metaplasia in salivary gland tumors and necrotizing sialometaplasia has occasionally been

reported (10-12), and Goulart et al. described that squamous metaplasia was derived from ductal

epithelium origin in the report. Immunohistochemically, the evidence of ductal epithelium origin had described that high molecular weight cytokeratins which revealed in the regular squamous epithelium were positive in the squamous metaplasia areas, and low molecular weight cytokeratins; CK7, CK19, and p63 were positive in the transitional area between hyperplasia and squamous metaplasia, respectively. And these immunohistochemical findings suggest that squamous metaplasia is relevant to the salivary gland tumor (10). On the contrary, reports about squamous metaplasia in otherwise the regular salivary ducts have been rare (13).

In the present study, to observe the process to squamous metaplasia, we investigated ultrastructural features of morphological alterations of the salivary ducts. In addition, we performed IHC staining to determine the expression of cytoskeletal and intercellular adhesion molecules associated with morphological alterations of the salivary ducts.

### *1. Clinicopathological findings*

Sialadenitis may be caused by bacterial infection, viral infection, autoimmune disease, or as

a complication of radiotherapy. Sialadenitis often occurs in the major salivary glands, commonly in the parotid gland, but also occurs in the submandibular gland and minor salivary glands. Although no sex difference in chronic sialadenitis has been reported, our study found that 75 of all 117 patients were women; thus, the percentage of women was significantly higher. One reason was probably because the excised surgical specimens in our study were from patients clinically diagnosed with chronic sialadenitis and sialolithiasis, and they included numerous lip biopsy specimens from patients with Sjögren syndrome, which has a predilection in women.

Sialolithiasis commonly occurs in the submandibular gland, and it may occur at all ages, but there is a peak incidence in the 4th, 5th and 6th decades. A higher incidence in men, but no sex difference, has been reported (14-18). Among the 55 patients in our study with both chronic sialadenitis and sialolithiasis, the most common site was the submandibular gland (39 patients), mean age was 60.6 years, and the male-female ratio did not significantly differ. This trend is in agreement with previous studies (15, 17, 18). Squamous metaplasia of the salivary ducts

develops due to abnormal chronic irritation. In our study, squamous metaplasia was observed in 31 (56.4%) of the 55 patients with both chronic sialadenitis and sialolithiasis. However, no significant difference was observed in the characteristics of the ductal epithelium based on presence or absence of sialolithiasis.

## *2. Histopathological, ultrastructural and immunohistochemical findings*

### *1) Ductal morphological findings*

Histological examination of the ducts in patients with chronic sialadenitis showed dilated ducts in which a bilayer was preserved, hyperplastic duct due to basal cell-like cells stratified, and squamous metaplastic ducts. Among all 117 patients, 22 had hyperplastic ducts, and 34 had squamous metaplasia. Thus, squamous metaplasia was observed in about 30% of these patients. In addition, frequent findings in patients with squamous metaplasia included a transition from hyperplasia to squamous metaplasia within the same ducts, and sloughing of the metaplastic squamous cells within ducts. Ultrastructurally, the lumen of the regular salivary gland ducts were



composed of cuboidal ductal cells. The hyperplastic ducts consisted of small cuboidal cells with high nuclear/cytoplasmic ratio and these cells thought to be stratified hyperplastic basal cells, because these small cells showed undifferentiated appearance without ductal and/or myoepithelial characteristics. Concerning about the squamous metaplastic region, it displayed squamous cell characters; centrally oriented nuclear, flattened cytoplasm, rich tonofilament bundles and desmosomal jointment.

## *2) Basal cell proliferation*

Morphological analysis of the hyperplastic ducts showed hyperplasia of cuboidal basal cell-like cells with relatively high nucleus/cytoplasmic (N/C) ratio. IHC staining was positive for p63, CK34 $\beta$ E12, CK5/6, CK19, and E-cadherin, thus reflecting basal cell characteristics. In addition, S100 was negative, thus negating a myoepithelial cell origin. The mechanism of metaplasia involves the degeneration of columnar epithelium due to physical irritation and pathogenic factors, which then leads to new cell proliferation and differentiation. In brief, this is

"not a change in already-mature cell characteristics," but rather a "change towards differentiation of proliferating cells" (2) or "reprogramming to new differentiation of stem cells" (3).

Regarding proliferating cells in the salivary glands, reserve cells exist for columnar cells in intercalated ducts, and for basal cells in excretory ducts. Each individual reserve cell is pluripotent and can differentiate into a basal cell or myoepithelial cell. This is a factor of proliferation and tumorigenesis in salivary gland (19-21). Reserve cell proliferation is also involved in squamous metaplasia (8); however, it is now commonly believed that all salivary gland cells have proliferative capacity (22, 23).

Ihrler et al. (13) stated that basal cells in excretory ducts are involved in ductal squamous metaplasia. In addition, Ihrler et al. (13) stated that basal cells play the central role for several directional morphogenetic differentiation in the majority of ductal metaplasia. In our study, basal cell hyperplasia was observed in the excretory ducts. Therefore, our findings are in disagreement with the reserve cell theory, namely, that only columnar cells in the intercalated

ducts have proliferative capacity (19-21). Additionally, Ihrler et al. (13) stated that basal cell hyperplasia is also associated with hyperplastic ducts, but did not mention how this hyperplasia occurs.

### *3) Activation of cell adhesion factors in basal cell hyperplasia*

E-cadherin is a transmembrane protein and epithelial intercellular adhesion molecule (24). E-cadherin is not only involved in intercellular adhesion, but also plays a role in cell polarity, cell differentiation, and tissue development (25-28).  $\beta$ -catenin binds to the cytoplasmic domain of cadherin on the cell surface membrane and is an intracellular molecule that functions to link cadherin to actin filaments (29). Concerning about the salivary glands, it was mentioned that the role of E-cadherin was a regulation of ductal lumen formation (29).  $\beta$ -catenin is involved in cell differentiation and polarization, and it also plays a role in the process of cell motility (30, 31).

Our study showed positive staining for E-cadherin in basal cells and columnar cells in the regular salivary ducts. These results are similar to previous reports (29, 32). In dilated ducts,

compared to regular ducts, a more positive reaction was observed for E-cadherin and  $\beta$ -catenin in columnar cells. This finding suggests a response to prevent a leakage of secretions (33). In hyperplastic ducts, there was a positive reaction in all layers. In immature squamous metaplastic ducts, staining was progressively more positive from the hyperplastic cells to the superficial cells.

E-cadherin plays an important role in maturation of ductal epithelial cells and in assembly of luminal structures. Specifically, E-cadherin is essential for survival of ductal epithelial cells during lumen formation (29). Therefore, in hyperplastic ducts and immature squamous metaplastic ducts, ductal basal cells were proliferated in response to physical irritation, and by stimulation of E-cadherin and  $\beta$ -catenin, the most important binding molecule with E-cadherins, maturation of proliferating basal cells and lumen formation had occurred. Furthermore, activation of  $\beta$ -catenin induces squamous metaplasia of duct epithelium. This has been reported in the breast (6) and prostate gland (34). Therefore, in salivary ducts, which also consist of columnar epithelium, stimulation of  $\beta$ -catenin probably induces ductal hyperplasia and

squamous metaplasia. In mature squamous metaplastic ducts, E-cadherin and  $\beta$ -catenin no longer serve these purposes and were only minimally expressed.

#### *4) Distribution of cytokeratin*

CK18 is a cytokeratin that is expressed in secretory epithelial cells that comprise the glandular epithelium (35, 36), CK19 is expressed in glandular epithelium and non-keratinized squamous epithelium (37, 38), CK5/6 is expressed in glandular epithelium stem cells (39), and CK34 $\beta$ E12 is expressed in ductal basal cells (37, 40, 41). In our study, in hyperplastic ducts, mainly superficial layer retained glandular epithelial characteristics and was positive for CK18. In the proliferating basal cells, CK5/6 and CK34 $\beta$ E12 were positive. In immature squamous metaplastic ducts, positive reaction for CK19, CK5/6 and CK34 $\beta$ E12 were attenuated. In mature squamous metaplastic ducts, characteristics of glandular epithelium were lost, and instead, CK13, which is a terminal differentiation marker in mucosal epithelium (35, 36), was expressed. From these results, the ductal epithelium was considered to have changed from glandular

epithelial type to mucosal type.

The above findings are similar to results for bronchial mucosa (8) and uterine cervical glands (42). In addition, our study also showed that expression of mitochondria, which are particularly abundant in striated ducts, was also strong in basal cell hyperplasia; however, this expression was attenuated with acquisition of squamous epithelial characteristics. These combined results suggest that basal cell hyperplasia is a squamous metaplastic process.

In conclusion, this study showed that dilation of ducts was due to abnormal physical irritation and that chronic irritation causes basal cell hyperplasia as a biological adaptive response. When this occurs, E-cadherin activation leads to basal cell maturation and lumen formation. On the other hand, activation of  $\beta$ -catenin, which is linked to this process, is associated with acquisition of squamous epithelium characteristics. Basal cell hyperplasia and squamous epithelium characteristics were also demonstrated by ultrastructural analysis by using electron microscopy. These changes are also supported by the fact that cytokeratin in ductal epithelial cells undergo transition from a glandular epithelial type to mucosal type.

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Figure 1

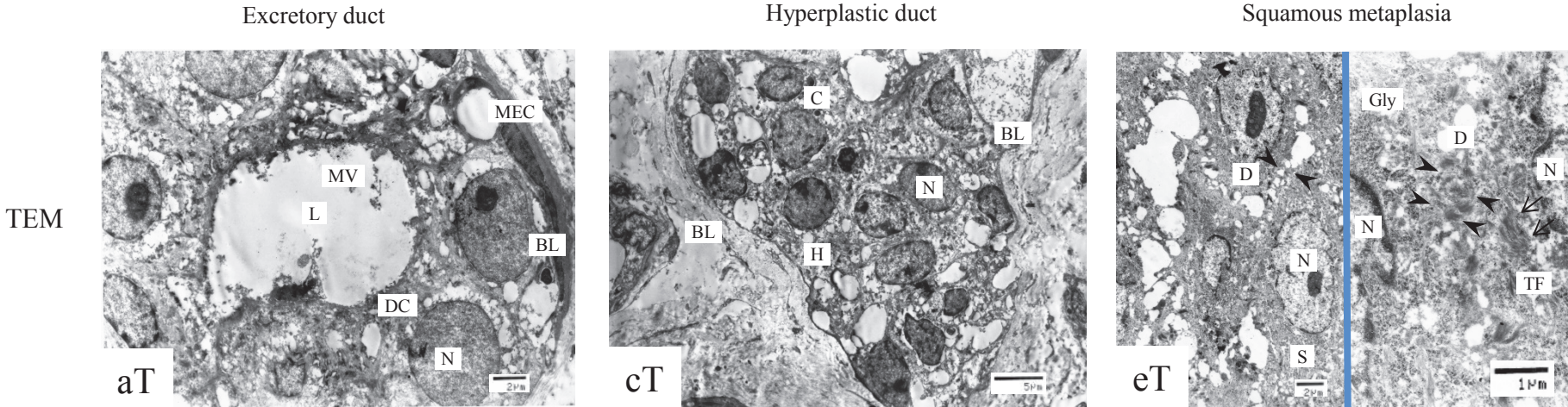
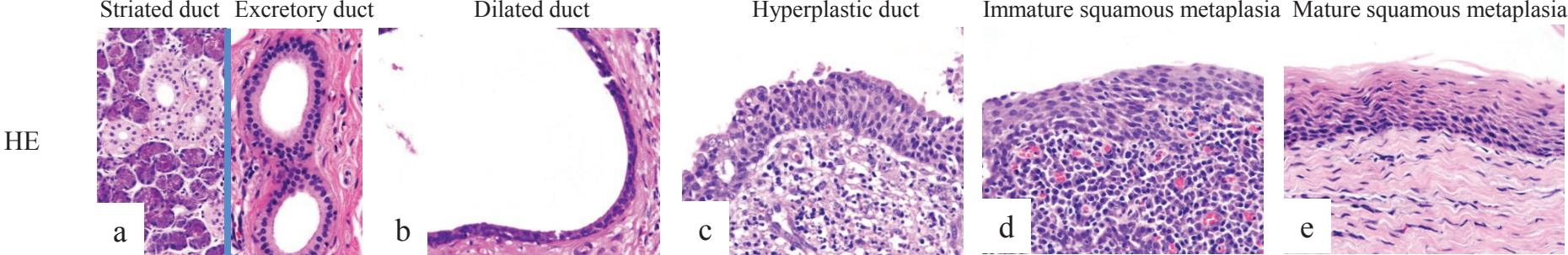
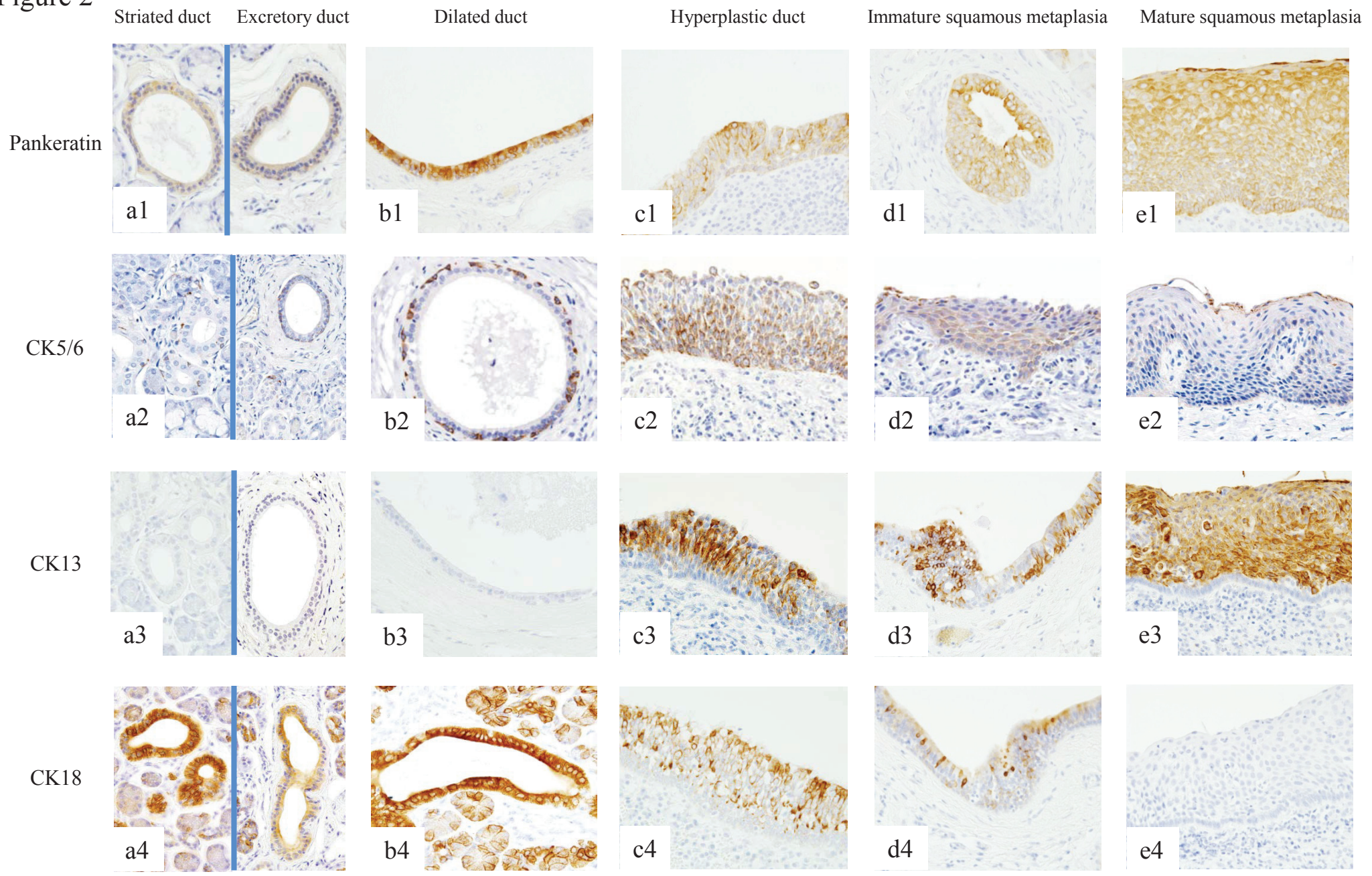


Figure 2





Striated duct

Excretory duct

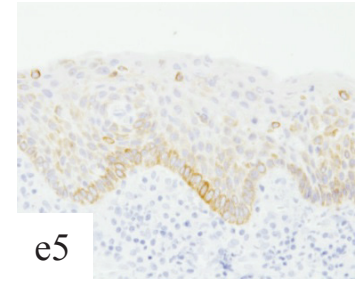
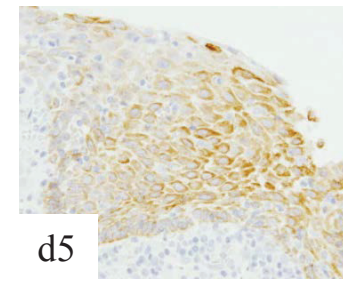
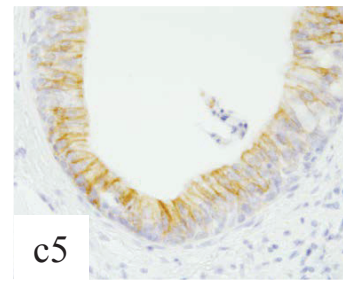
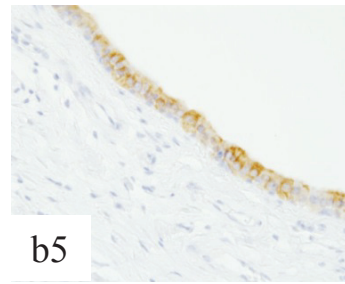
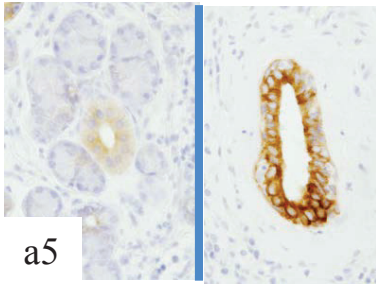
Dilated duct

Hyperplastic duct

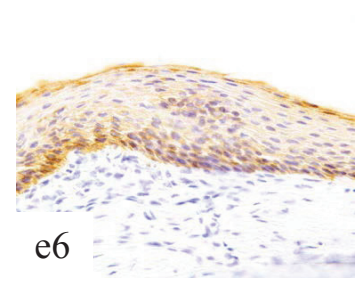
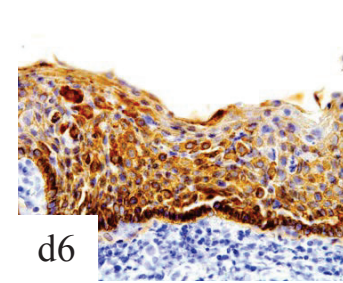
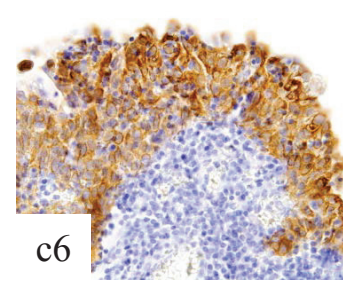
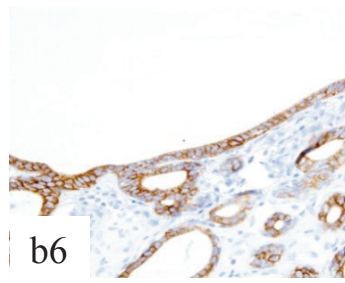
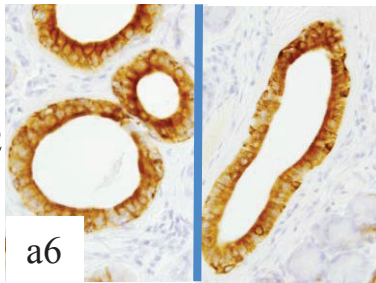
Immature squamous metaplasia

Mature squamous metaplasia

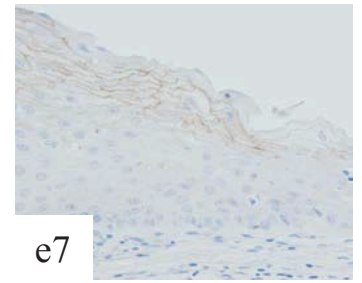
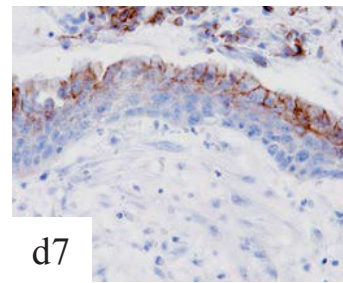
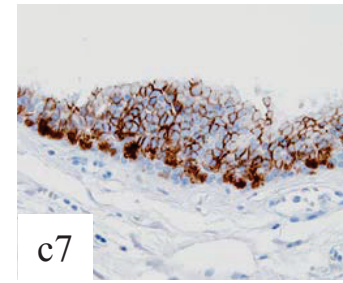
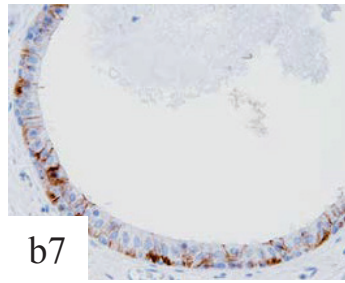
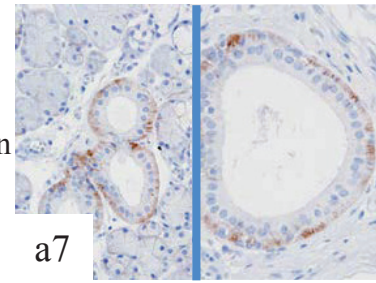
CK19



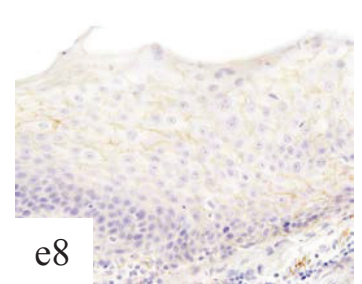
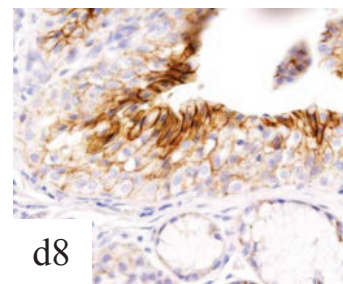
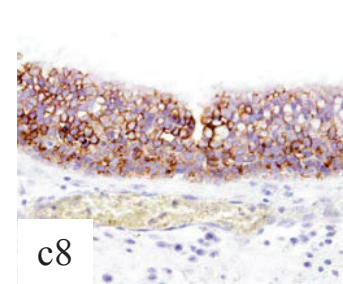
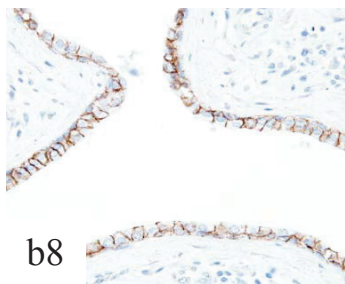
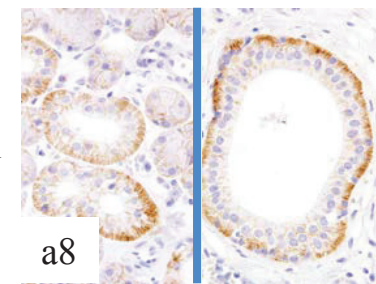
CK34 $\beta$ E12



E-cadherin



$\beta$ -catenin



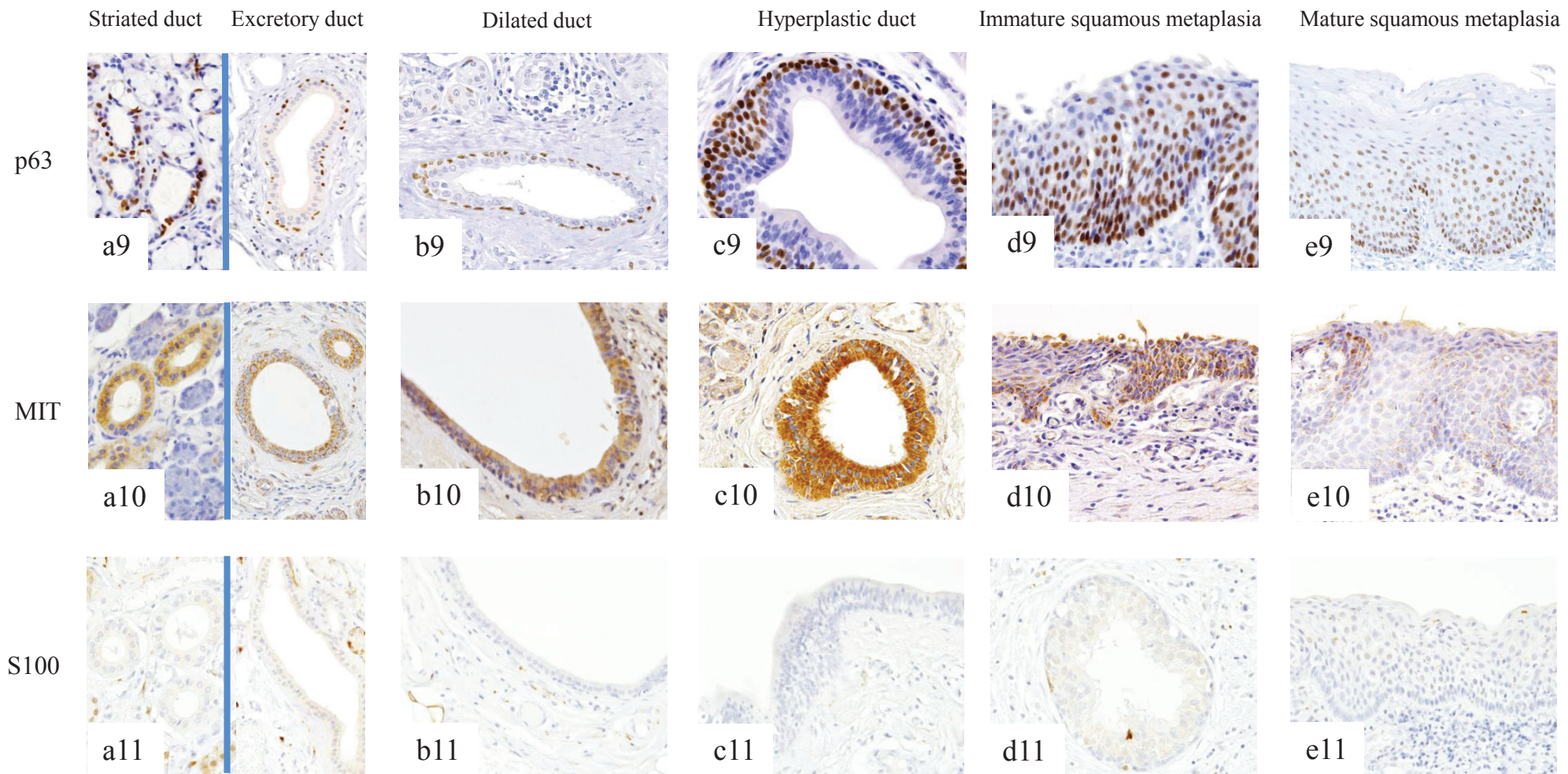
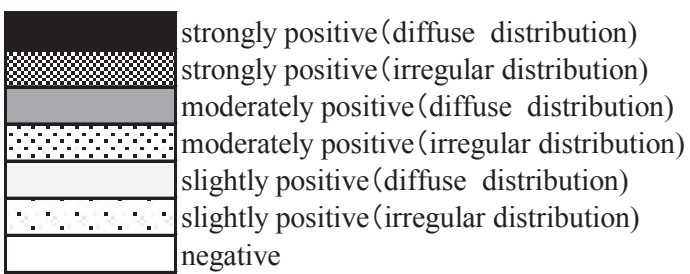
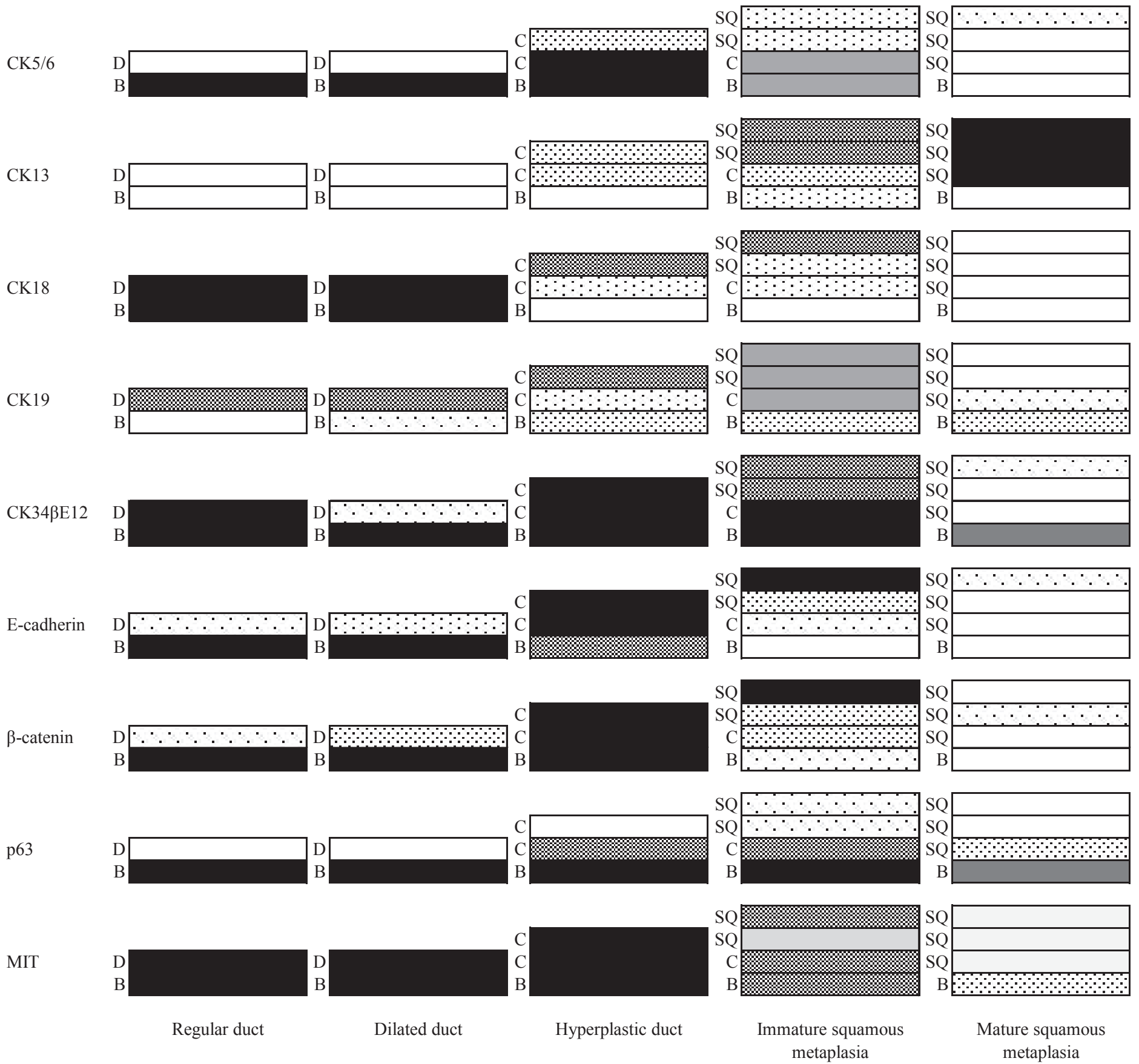


Figure 3



D ductal cells  
 B basal cells  
 C cuboidal cells (hyperplastic cells)  
 SQ squamous cells

## Figure legends

### Figure 1

Histopathological findings of regular duct (left: striated, right: excretory duct), dilated duct (b), hyperplastic duct (c), immature squamous metaplasia (d), and mature squamous metaplasia (e).

(Original magnification:  $\times 600$ )

Ultrastructural features of normal excretory duct (aT), hyperplastic duct (cT) and squamous metaplasia (eT). (aT) Microvilli-line (MV) lumen (L) of an intercalated duct is lined by cuboidal ductal cells (DC), and a myoepithelial cell (MEC) subjacent to the basal lamina (BL).

(cT) The stratified (hyperplastic) duct (H) consisted of small cuboidal cells (C) with high nuclear (N)/cytoplasmic ratio and mitochondria. (eT) The region of squamous metaplasia (S) is

composed of flattened cells with centrally oriented nuclear. These cells contain glycogen granules (Gly) and tonofilament bundles (TF) (arrows), and are joined by well-formed desmosomes (D) (arrowheads). (Original magnification: (aT);  $\times 2,500$ , (cT);  $\times 1,500$ , (eT)

left;  $\times 2,000$ , right;  $\times 8,000$ ).

## Figure 2

Immunohistochemical labeling for pankeratin (a1-e1), CK5/6 (a2-e2), CK13 (a3-e3), CK18 (a4-e4), CK19 (a5-e5), CK34βE12 (a6-e6), E-cadherin (a7-e7), β-catenin (a8-e8), p63 (a9-e9), MIT (a10-e10), S100 (a11-e11). (Original magnification: ×600)

All epithelial cells showed positive for pankeratin (a1-e1). CK5/6 showed strong positivity in basal cells and hyperplastic cells in hyperplastic ducts (c2). The mature squamous metaplastic ducts were negative for CK5/6, except for some minimal expression in the superficial cells (e2). CK13 showed negative reaction in dilated duct (b3) and increased positivity from immature (d3) to mature metaplasia (e3). CK18 was negative in mature squamous metaplastic ducts (e4). For CK19 in hyperplastic ducts, moderate positivity in hyperplastic cells and strong positivity in columnar-like superficial cells were observed (c5). CK34βE12 showed strongly positive cells were evident in all layers in hyperplastic ducts (c6). In mature squamous metaplastic ducts, the basal cells were moderately positive, and the superficial cells were weakly positive for

CK34βE12 (d6). E-cadherin and β-catenin showed positivity in all layer in hyperplastic ducts (c7 and c8). In mature squamous metaplastic ducts, E-cadherin and β-catenin were minimally expressed (e7 and e8). In hyperplastic ducts, there was strong positivity in almost all basal cells, and strong positivity in hyperplastic cells for p63 (c9). Strong positivities in all layers of MIT were observed in dilated and hyperplastic ducts (b10 and c10) and positivity was decreased from immature to mature squamous metaplasia ducts (d10 and e10). S100 showed all cells in the dilated ducts, hyperplastic ducts, and squamous metaplastic ducts were negative for S100 (a11-e11).

### Figure 3

Schematic representation of the immunohistochemical distribution patterns as detected by the different antibodies. Filled bars indicate a diffuse positive reaction, black bar means a strongly reaction, gray bar means a moderately reaction, light color bar means slightly reaction. Open bars indicated a negative reaction. A stippled bar indicate an irregular positive reaction, thick

stippled bar means a strongly reaction, common stippled bar means a moderately reaction, thin

stippled bar means slightly reaction.

Abbreviations: D=ductal cells; B=basal cells; C=cuboidal cells (hyperplastic cells);

SQ=squamous cells.

Table 1 Clinical summary

Histological Diagnosis	Histological change of duct	Total number	Average age±SD	Region			
				Submandibular gland	Sublingual gland	Parotid gland	Minor salivary glands
Sialolithiasis and chronic sialadenitis	Non*	9(M:2 , F:7)	60.6±19.8	4	0	0	5
	Hyperplasia	15(M:5 , F:10)	54.6±18.7	10	1	1	3
Chronic sialadenitis	Squamous cell metaplasia	31(M:19 , F:12)	56.5±15.0	25	0	1	5
	Non*	52(M:15 , F:46)	53.6±18.0	1	2	0	49
	Hyperplasia	7(M:1 , F:6)	66.9±27.9	1	1	0	5
	Squamous cell metaplasia	3(M:2 , F:1)	58.7±28.8	1	0	0	2
Total		117(M:42 , F:75) **	56.0±17.4	42	4	2	69

M: male

F: female

SD: Standard Deviation

\*Non: the regular salivary duct (including dilated duct)

\*\*Kruskal-Wallis test (p<0.001)



Table 2 Subjects of immunohistochemical study

Histological diagnosis	Case No.	Age	Sex	Region
Chronic sialadenitis	1	70	Female	Lower lip
	2	70	Female	Lower lip
	3	68	Female	Lower lip
	4	67	Female	Lower lip
	5	55	Female	Lower lip
	6	54	Female	Lower lip
	7	53	Female	Lower lip
	8	50	Female	Lower lip
	9	48	Female	Lower lip
	10	45	Female	Lower lip
	11	44	Female	Lower lip
	12	36	Female	Lower lip
	13	63	Male	Upper lip
	14	75	Male	Cheek
	15	45	Male	Cheek
Sialolithiasis and chronic sialadenitis	1	73	Male	Submandibular gland
	2	53	Male	Submandibular gland
	3	49	Male	Submandibular gland
	4	28	Male	Submandibular gland
	5	67	Female	Submandibular gland
	6	65	Female	Submandibular gland
	7	62	Female	Submandibular gland
	8	61	Female	Submandibular gland
	9	58	Female	Submandibular gland
	10	80	Male	Cheek