学 位 論 文

Notch as a Possible Cell Differentiation Factor in Pleomorphic Adenomas

（多形腺腫における細胞分化因子としてのNotchの可能性）

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松本歯科大学大学院歯学独立研究科博士（歯学）学位申請論文

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《要旨》

【緒言】多形腺腫は良性上皮性腫瘍に分類される唾液腺腫瘍の中でも最も一般的なものである。腫瘍組織内には様々の細胞種がみられることが知られており、その細胞分化については興味深い事象である。本研究では細胞の分化・増殖に関与することが知られている Notch が多形腺種の多様な細胞分化に関しても強く関与しているとの仮説を立て、Notch の分布状況を検討し、若干の結果を得たのでここに報告する。

【材料と方法】研究材料は、愛知学院大学歯学部口腔病理学講座にて取り扱われ、多形腺腫と診断された外科病理ファイルの中から病理組織学的に再評価を行い、WHO の分類に基づく典型的な多形腺腫 30 症例（平均年齢は 51.5 歳、男性 13 症例、女性 17 症例）である。Notch による免疫組織化学的検討を加えた後、CK7 と 13 との免疫蛍光染色による重ね合わせを行い、検討した。

【結果】病理組織学的には、大小の空隙がみられるなど様々な組織構造の存在する多彩性を示していた。類円形を示す腫瘍実質部分は線維性の比較的薄い被膜に覆われており、被膜の内部には線維性組織の中に腫瘍胞巣の増殖が確認できた。腫瘍内の構成細胞は、随所に小型円形ないし嚢胞状の腺腔を形成する導管上皮細胞とその周囲に位置する位置する筋上皮細胞の主として2種類の増殖からなっていた。間葉様部分には筋上皮細胞の増殖からなり、これが次第に粗になって形成され、紡錘形、類円形ないし楕円形細胞が腫瘍胞巣から解離増殖することによって形成されたいわゆる“mixed appearance”像、それに続いて粘液腫様組織、さらに一部では軟骨様組織も特徴的に観察することができた。また、充実性に増殖する腫瘍細胞が扁平上皮へと化生し組織内に著しく角質変性を起こしている部位もみられた。免疫組織化学的には Notch は、腺腔構造を形成する導管上皮細胞の細胞質に陽性反応があり、一部の細胞の核に陽性反応がみられた。その周囲の充実性に増殖した腫瘍細胞の多くにも核内に陽性反応が存在していた。また、扁平上皮化生部における基底細胞様細胞層の細胞質が強い反応を示し、核にも一部の細胞で反応があった。核内反応および細胞質の反応は有棘細胞様細胞層へ向かうに従い弱まる傾向が確認できた。腺管様の構造を形成する導管上皮細胞は細胞骨格である CK7 に陽性であった。同部について、免疫蛍光染色により、Notch は腺腔を形成する導管上皮細胞内の一部の核に反応を呈した。またこの反応が確認できた部分は CK7 の存在する部位であり、その周囲に存在する充実性に増殖した多くの腫瘍細胞にも Notch の反応が認められた。Notch と CK7 の重ね合わせにより Notch は腺腔構造を形成している導管上皮細胞の周囲に反応が観察された。また、腺腔構造を形成する導管上皮細胞の一部に核内に反応があった。同じく細胞骨格である CK13 に染色された扁平上皮化生様部分を観察したところ、CK13 は扁平上皮化生を生じた腫瘍細胞の有棘細胞様細胞を中心に陽性反応を示した。一方で、Notch は基底細胞様細胞部から有棘細胞様細胞部にかけて核内に存在していた。CK13 と Notch を重ね合わせると、扁平上皮化生部の中でも基底細

胞様細胞部の細胞の多くが Notch の陽性を示し、表層に向かうに従ってその数は減少していくことわかった。また、これらを重ね合わせて核内反応を観察してみると、基底細胞様細胞の核内に反応がみられ、有棘細胞様細胞層へと向かうに従い、核内陽性反応を示す細胞が少なくなっていることが観察できた。多形腺腫における特徴的な構造物である軟骨様部分は細胞質が強い反応を示したが、核に反応はなかった。同じく、特徴的な構造物である結合性を喪失したことにより生じた粘液腫様の組織部分は、一部の腫瘍性筋上皮細胞の細胞膜に若干の反応を示したが、細胞質および核に反応は認められなかった。

【考察】多形腺腫の腫瘍実質部分で CK7 に陽性反応を示している導管上皮細胞部を Notch と比較することにより、一部の細胞の核内に陽性反応がみられることが明らかとなった。腺腔構造部の周囲に存在する充実性に増殖した腫瘍細胞の核内にも陽性反応があったことから腺管構造部における導管上皮細胞における細胞分化に Notch が関与し、まさに分化途上ということが示唆される。CK13 にて染色を行った扁平上皮化生部については、Notch は基底細胞様細胞の核内から有棘細胞様細胞の核に反応がみられた。これが表層に向かうに従い核内に反応を示す細胞が少なくなっていったことから、基底細胞様細胞層の時点においては分化の最中であることが示唆され、有棘細胞様細胞へと基底細胞様細胞の分化が進んでいる。表層に向かうに従い、核内の陽性反応が弱くなっていることから、分化が完了に向かっていることが考察できる。また、多形腺腫に特徴的な構造である物である軟骨様部分、粘液腫様の組織部分は、核に反応がみられなかったため、それぞれの構造物へと分化が進み終わり、現在は分化が止まっている状態であると考察した。以上の事より、本研究から多形腺腫の多彩な細胞分化には部位により Notch が大きく関与していることが示された。

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Research Paper

Notch as a Possible Cell Differentiation Factor in Pleomorphic Adenomas

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Abstract

The expression of Notch in 30 cases of pleomorphic adenoma was examined by immunohistochemistry. Comparing the results of our study with previous literatures, from the partial CK7 expression and substantial Notch expression in ductal epithelial cells as well as the Notch expression in solid tumor nests, it can be inferred that Notch is involved in cell differentiation. CK13 expression was observed in cells undergoing squamous metaplasia and Notch expression was seen in the nucleus of basal and squamous cells. The intense Notch expression in basal cells and weak expression in squamous cells suggests that Notch is involved in the differentiation from basal to squamous cell. Moreover, the loss of nuclear expression on the inner layer would signify that differentiation is about to end or has been terminated. Notch was expressed in the cytoplasm of cartilage cells and in the cell membrane of mucous cells but not in the nucleus indicating that differentiation has been concluded. Notch involvement is suspected in cell differentiation in areas showing ductal structures and squamous metaplasia. In summary, Notch is involved in cell differentiation of ductal cells in PA. Nuclear expression was shown in tumor cells in solid nests and surrounding structures. Moreover, Notch is expressed by basal cells undergoing squamous metaplasia suggesting the participation of Notch in cell differentiation in PA.

Key words: Notch1, pleomorphic adenoma, cell differentiation, immunohistochemistry

Introduction

Pleomorphic adenoma (PA) is the most common salivary gland tumor classified as benign epithelial tumors (1). Various cell types can be seen in the tumor indicating a high occurrence of cell differentiation. Okuda et al. performed immunohistochemistry on PA to determine the role of Wnt in cell differentiation (2). The results suggested that Wnt is involved in cell differentiation in PA. In the present study, we considered that Notch might also be involved in cell proliferation and differentiation in the same manner that Okuda et al. did on Wnt (3).

In this study, we focused on Notch expression in PA since it has been hypothesized to be strongly associated with neoplastic cell differentiation. Moreover, studies on the expression of Notch have been tremendously increasing.

Materials and Methods

Tissue samples from Department of Oral Pathology, School of Dentistry, Aichi Gakuin University, diagnosed histologically as PA were further re-evaluated for the purpose of this research. A total of 30 cases of PA based on WHO classification were used in this experiment. The specimens used in this study were the same as those used by Okuda et al. (2), described in Table 1.

Specimens were fixed in neutral buffered formalin solution, subjected into series of alcohol for dehydration and then embedded in paraffin. Then after, the specimens were serially sectioned into 4 μm

stained with hematoxylin and eosin and examined under a light microscope.

Table 1. Cases examined

Age		Sex		Location	
Average	51.5	Male	13	Palate	14
		Female	17	Parotid gland	5
				Mandibular gland	4
				Upper lip	3
				Buccal mucosa	3
				Other	1

Immunohistochemistry was performed in the following manner. Briefly, deparaffinized sections were pre-treated for antigen retrieval in citrate buffer (Mitsubishi Chemical Medience, Tokyo, Japan) with a pH of 6.0 and placed in autoclave at 120 °C for 15 min. Then after, sections were covered with serum-free protein block (Dako Japan Co., Ltd., Tokyo, Japan), incubated at room temperature for 30 min. Notch1 rabbit polyclonal antibody (Anti-Notch-1 intracellular domain antibody, ab83232, Abcam, Cambridge, UK) was used as the primary antibody. This was followed by staining with Dako Chem Mate Envision Kit (Dako). Antigenic sites were revealed using DAB.

Immunofluorescent staining was also performed using Notch1 rabbit polyclonal antibody, CK7 mouse monoclonal antibody (1:100; Abcam) and CK13 mouse monoclonal antibody (AE8; 1:100; Abcam). Double staining was done by combining Notch1 and CK7 as well as Notch1 and CK13. After deparaffinization, Notch1-CK7 and Notch1-CK13 were pre-treated in citric acid buffer (Mitsubishi Chemical Medience, Tokyo, Japan) with a pH of 6.0 and placed in microwave for 1 min. Then after, sections were covered with serum-free protein block (Dako), incubated at room temperature for 30 min. The primary antibodies Notch1 and CK7 (1:100; Can Get Signal, Toyobo Co.,, Osaka, Japan) were allowed to react at 4 °C for 16 hours. For the secondary antibody, Donkey anti-rabbit IgG H&L (1:200; Alexa Fluor 594; Abcam) and Donkey anti-mouse IgG H&L (1:200; Alexa Fluor 488; Abcam) were carried out after reaction with Can Get Signal (Toyobo) at 1:200 at room temperature for 60 min. DAPI was allowed to react for 3 min for nuclear staining. The present study was approved by the ethics committee of Aichi Gakuin University, School of Dentistry under the title "Diagnosis and Clinicopathological Study on the Elucidation of Salivary Gland Tumors' (No. 284, December 5, 2011).

Results

Histopathological examination

Generally, the tumor consists of various tissue types with small and/or large duct-like/cystic spaces in the tissues. The tumor is round in shape and covered with relatively thin fibrous connective tissue. The substantial portion of the tumor consists of glandular structures of tumor nests surrounded by fibrous tissue. The tumor is clearly separated from the normal salivary gland tissues. The tumor stroma consists of duct-like structures predominantly ductal and myoepithelial cells forming cystic cavities.

The mesenchymal part consists of myoepithelial cells, spindle shaped cells and myxoma-like tissue formed by round or oval cells dissociated from the tumor growth. In some areas, cartilage-like tissues can be characteristically observed (Fig. 1-a). In myxomatous areas, the myoepithelial cells have sparsely formed cord-like structures and the presence of mucous was confirmed. Cartilage-like tissues can be seen in a wide range possibly due to the presence of cartilage-like matrix and cartilage-like cells. Neoplastic myoepithelial cells are dissociated from the mucous or cartilage-like substrate by their own secretions. The tumor parenchyma might have been formed by myxomatous cells, which migrated to seep into the structure of mesenchymal and cartilage-like cells. Thus, the epithelial component is dissociated and mixed into the mesenchymal-like tissue, giving rise to a 'mixed appearance'. Moreover, tumor cells undergo remarkable squamous metaplasia (Fig. 1-b).

Immunohistochemical findings

Notch1 expression was observed in the cytoplasm of ductal epithelial cells as well as in some of the nuclei. Notch expression was also observed in the nucleus of tumor cells surrounding solid tumor nests (Fig. 1-c).

Notch was expressed in the cytoplasm of plasmacytoid myoepithelial cells but not in the nucleus. In areas undergoing squamous metaplasia, Notch was strongly expressed in the cytoplasm and nucleus of basal cells. However, the expression became weak towards the outer squamous cells (Fig. 1-d).

The cartilage-like cells representing the characteristic feature of PA showed strong cytoplasmic expression but not nuclear reaction (Fig. 1-e). Likewise, the myoepithelial cells in myxomatous area showed faint expression on the cell membrane but no cytoplasmic or nuclear reaction were detected (Fig. 1-f).

Ductal epithelial cells expressed CK7 (Fig. 2-a). Notch was also detected in the nucleus of some ductal epithelial cells (Fig. 2-b). Double immunofluorescent staining revealed that Notch and CK7 were co-expressed by tumor cells in ductal structures. Notch expression was observed in most of the nucleus of tumor cells in solid nests as well as those in ductal structures (Fig. 2-c, d).

The expression of CK13 in tumor cells undergoing squamous metaplasia was also observed. CK13 showed positive reaction on basal and prickle cell layers (Fig. 3-a). On the other hand, Notch was observed in the nucleus from basal cell to squamous cells (Fig. 3-b). Double immunofluorescent staining of Notch and CK13 revealed that most basal cells expressed Notch although expression decreased towards the squamous cells. Furthermore, nuclear expression was observed in basal cells and the expression became weak on the prickle cell layer (Fig. 3-c, d).

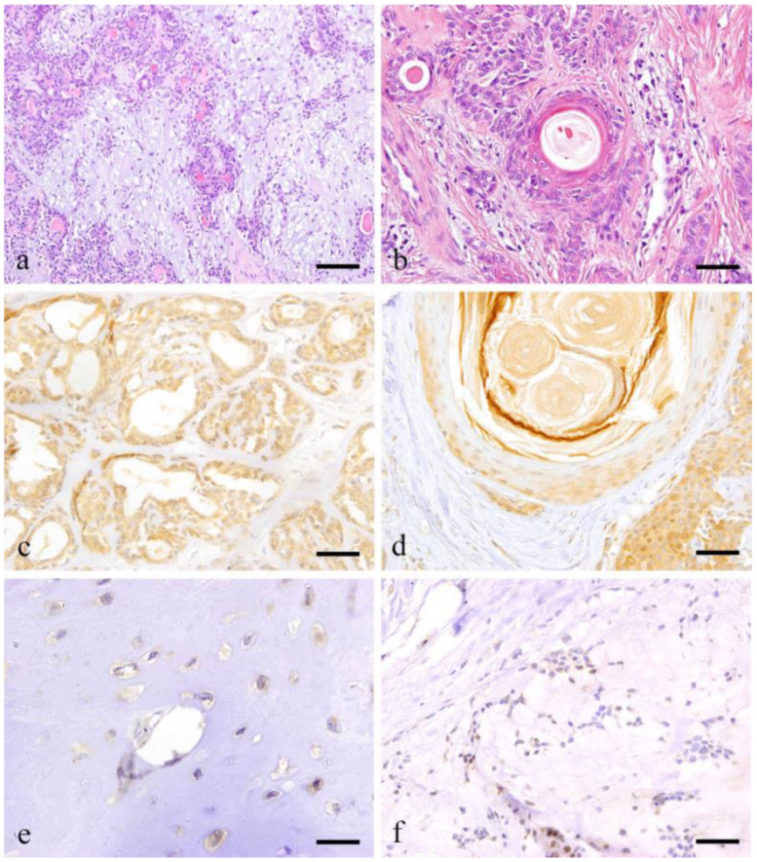

Figure 1. Histopathologcal features of typical PA (a: Case 9: 28 year-old female, the right palatal region; scale bar=200μm), squamous metaplasia with keratin pearl (b: Case 30: 40 year-old male, left palatal region; scale bar=100μm), immunohistochemical positive reactions in ductal areas (c: Case 2: 40 year-old female, right palatal region; scale bar=100μm), in squamous metaplasia area (d: Case 18: 69 year-old female, right buccal mucosa; scale bar=100μm), immunohistochemical features of cartilage-like area (e: Case 18: 69 year-old female, right buccal mucosa; scale bar=20μm) and myxomatous area (f: Case 18: 69 year-old female, right buccal mucosa; scale bar=50μm).

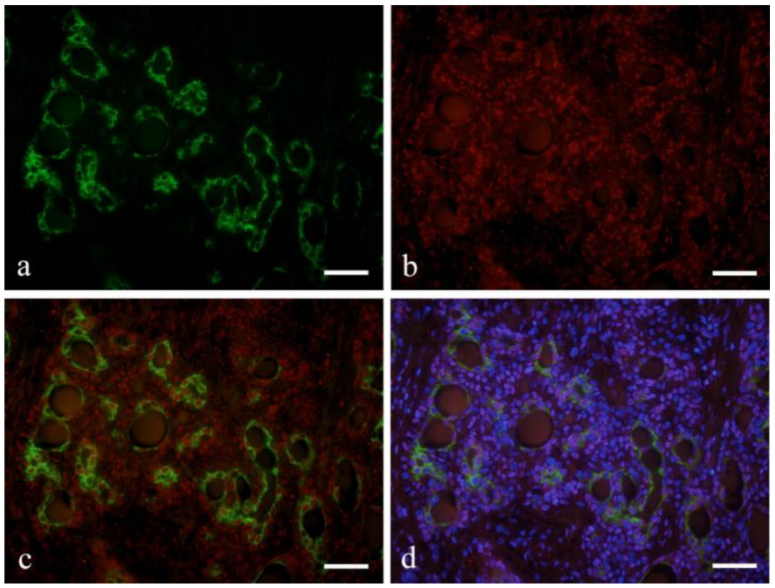

Figure 2. Immunofluorescent staining images of ductal structure forming area (a: CK7; b: Notch1; c: merged image of CK7 and Notch1; and d: merged image of CK7 and Notch1 and DAPI; Case 28: 39year-old male, submandibular region; scale bar=100μm).

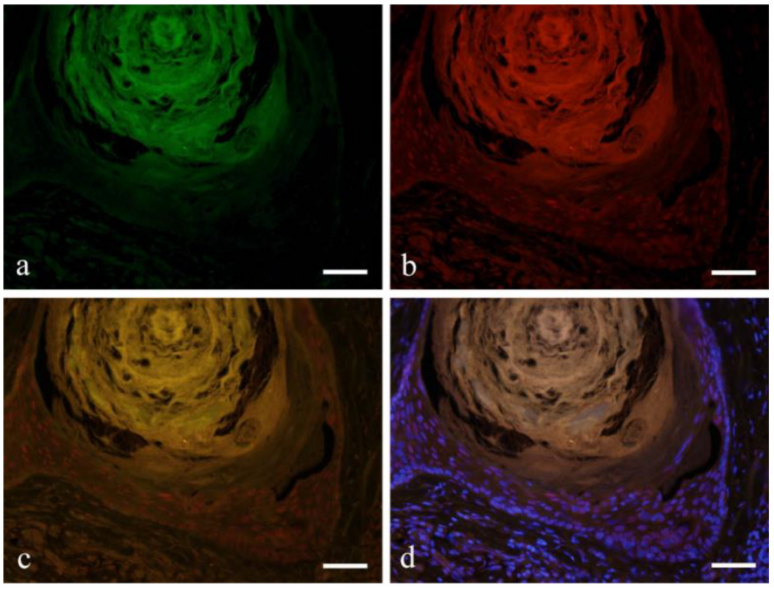

Figure 3. Immunofluorescent staining images of squamous metaplasia area with keratin pearl (a: CK13; b: Notch1; c: merged image of CK13 and Notch1; and d: marged image of CK13 and Notch1 and DAPI; Case 18: 69 year-old female, right buccal mucosa; scale bar=100μm).

Discussion

PA is the most common benign epithelial tumor of salivary glands based on WHO classification (1). The tumor shows a wide variety of tumor development brought about by cell proliferation and differentiation as mentioned in previous studies (4-6).

Okuda et al. studied the expression of Wnt in 30 cases of PA. The results showed that tumor cells in ductal structure and in solid tumor nests expressed Wnt. It was inferred that β-catenin pathway was involved in cell differentiation indicated by Wnt expressions in cells undergoing squamous metaplasia (2). For this reason, we believed that Notch would have a role in tumor cell differentiation considering its association with other tumors (7, 8).

Notch appears as a typical signal that controls the growth of tissues responsible for the fate of cells (9). Notch is a single-pass transmembrane receptor with a domain outside and inside the cell membrane. Notch intracellular domain (NICD) is cleaved and binds to a ligand such as Jagged. Cleaved NICD is moved from the cell membrane into the cytoplasm, binds with Suppressor of Hair-less (Su(H)) to form a complex and acts on downstream target gene in the nucleus thus activating the expression (10). In addition to the role of transmitting instructions for various morphogenesis and tissue differentiation during development, Notch plays an important role in cell-cell signaling and is involved in stem cell maintenance, differentiation and neuronal function in adult.

When there is a disturbance in the activity of Notch signaling pathway, it becomes oncogenic detected in several cancers such as esophageal cancer, breast cancer and lymphoblastic acute leukemia (T-ALL) (11, 12). In addition, Notch has been the focus of researches on the metastasis of malignant tumors such as adenoid cystic carcinoma and malignant ameloblastoma (13, 14), believed to be a huge factor in the progress of malignant tumors.

Studies in Notch have been done in odontogenic tumors such as ameloblastoma (15-17). Notch is expressed in solid nests and in squamous metaplasia in ameloblastoma, implicating its contribution in cell differentiation and morphogenesis. Moreover, in calcifying cystic odontogenic tumor, calcifying epitheioma (pilomatrixoma), odontoma and craniopharyngioma, Notch expression in ghost or shadow cells was observed (18-20). Notch overexpression in ghost cells inhibited the development of neighboring cells. This means that Notch inhibits the production of a ligand in receiving cell signal between Notch ligand and Notch receptor to adjacent cells through a negative feedback mechanism. In contrast, during binary cell fate determination, Numb acts an inhibitor of Notch signaling cycle causing asymmetric distribution of the Notch pathway. This led to overexpression suggesting that Notch-Jagged1 is the primary mechanism in determining the fate of ghost cell (21).

Squamous metaplasia depicted as having a variety of cell differentiation is commonly observed in odontogenic tumors such as ameloblastoma. Squamous metaplasia can also be observed in PA. Muraki et al suggested a close relationship between Notch and ameloblastoma shown by the localization of Notch in peripheral tumor nests (15). From this aspect, we suspected that Notch might also be involved in cell differentiation in PA.

Comparing the results of our study with previous reports, from the partial CK7 expression and substantial Notch expression in ductal epithelial cells as well as the Notch expression in solid tumor nests, it can be inferred that Notch is involved in cell differentiation (2, 22). CK13 expression was observed in cells undergoing squamous metaplasia and Notch expression was seen in the nucleus of basal and squamous cells (23). The intense Notch expression in basal cells and weak expression in squamous cells suggests that Notch is involved in the differentiation from basal to squamous cell. Moreover, the loss of nuclear expression on the surface layer would signify that differentiation is about to end or has been terminated. Notch was expressed in the cytoplasm of cartilage cells and in the cell membrane of mucous cells but not in the nucleus indicating that differentiation has been concluded.

Notch involvement is suspected in cell differentiation in areas showing ductal structures and squamous metaplasia. In the study by Okuda et al, small cuboidal cells forming ductal structure expressed Wnt and the expression was also confirmed in the basal cells surrounding those cells undergoing squamous metaplasia. The results coincided with our present study implicating the function of Notch in the same sites causing cell differentiation (2).

In summary, Notch is involved in cell differentiation of ductal cells in PA. Nuclear expression was shown in tumor cells in solid nests and surrounding structures. Moreover, Notch is expressed by basal cells undergoing squamous metaplasia suggesting the participation of Notch in cell differentiation in PA.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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