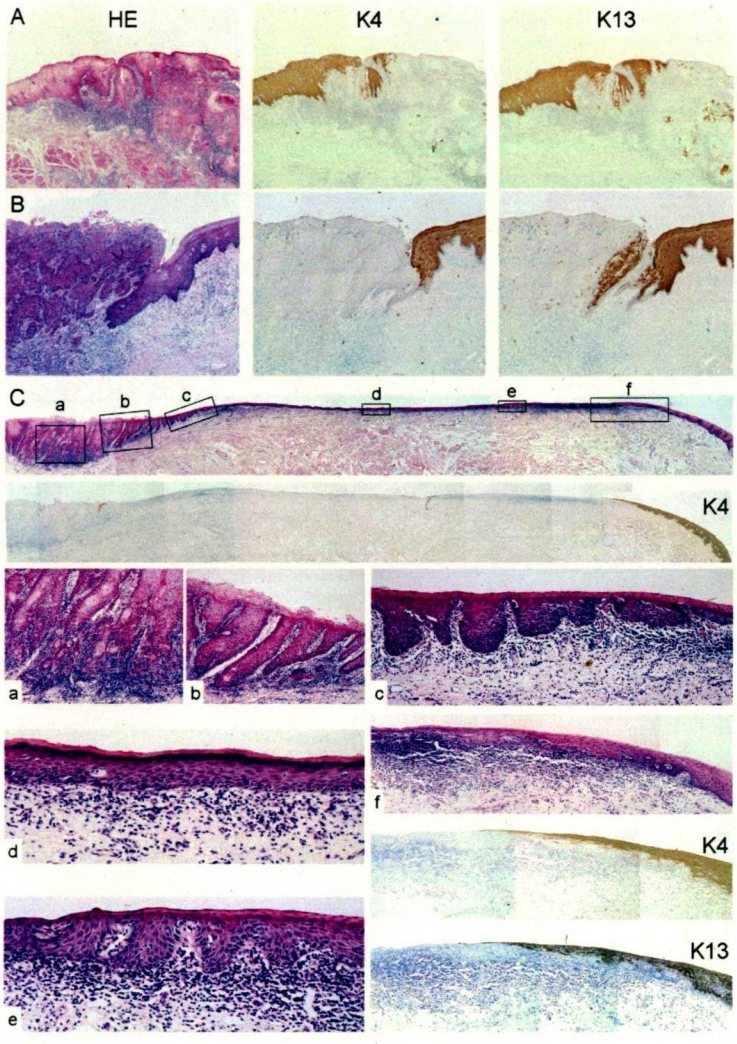

D

	OED			OSCC
	Mild	Moderate	Severe	
K4 ↓	16/16 (100%)	24/24 (100%)	10/10 (100%)	50/50 (100%)
K13 ↓	6/16 (38%)	19/24 (79%)	10/10 (100%)	49/50 (98%)

Figure 3. K4 and K13 expression in OSCC and OED. A) Absence of K4 and K13 expression in well-differentiated SCC of tongue. Most of the cancer cells were negative for both K4 and K13. Some cancer nests contained K4(+) or K13(+) cells in a scattered fashion, where K13(+) cells were observed more than K4(+) cells. B) Absence of K4 and K13 expression in early SCC of tongue. The small dysplastic lesion between invasive cancer and normal epithelium showed absent K4 expression, and remaining but downregulated expression of K13. C) A representative case of SCC associated with OED that was clinically observed as a mixture of erythroplakia and leukoplakia in the buccal mucosa. High magnification views of selected areas are shown (a-f). The lesion exhibited various histological appearances; a) Invasive squamous cell carcinoma. b) Hyperparakeratosis and acanthosis with irregularly elongated rete ridges. c) Weak keratinization and slightly bulbous rete ridges. d) Orthokeratinization with minimal architectural and cellular atypia. e) Weak keratinization with irregular shapes of rete ridges. f) The periphery of the lesion, revealed by the expression of K4 and K13 as well as by histology. On the left, the affected epithelium was thin with little tendency of

cellular keratinization. K4 and K13 were downregulated in all these lesions (a-f). D) Summary of the immunohistochemical examination. A number of cases with distinct downregulation of K4 or K13 are shown.
481x764mm (87 x 87 DPI)

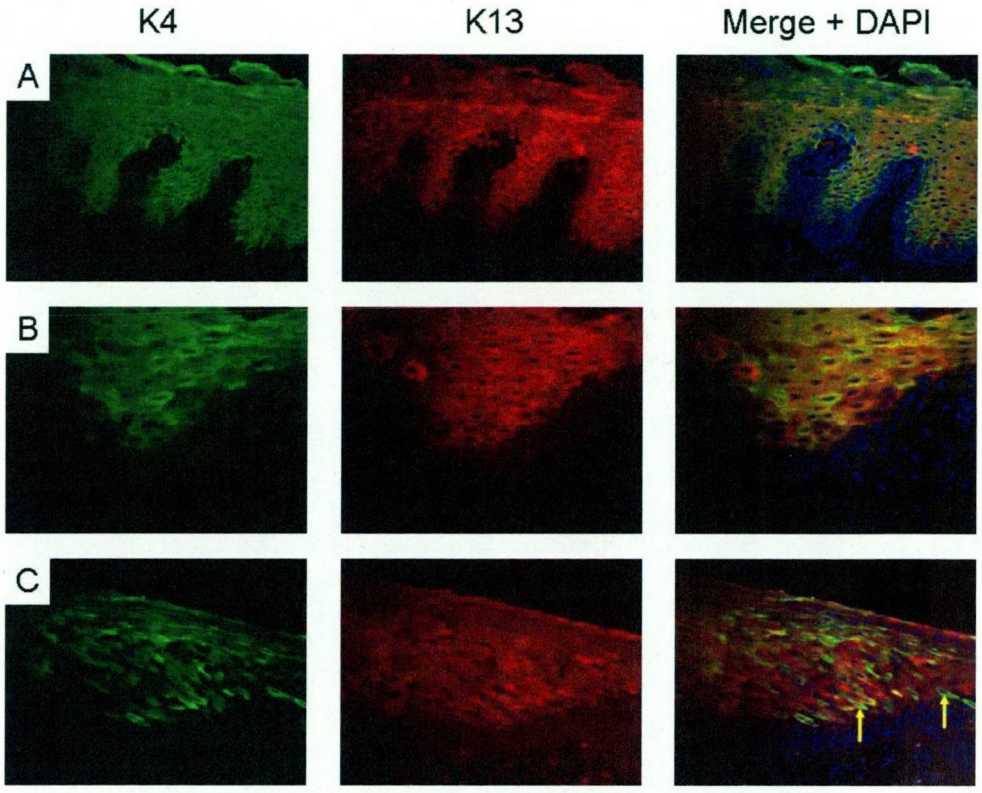

Figure 4. K4 and K13 expression in OED revealed by immunofluorescent double staining. A) Normal epithelium, whose suprabasal cells are K4(+)K13(+). B) Mild OED. K4(-)K13(+) cells are observed at the periphery of the lesion. C) Type 2 border of moderate OED. In this transient zone, a few K4(+)K13(-) cells are found (arrows).
250x204mm (96 x 96 DPI)

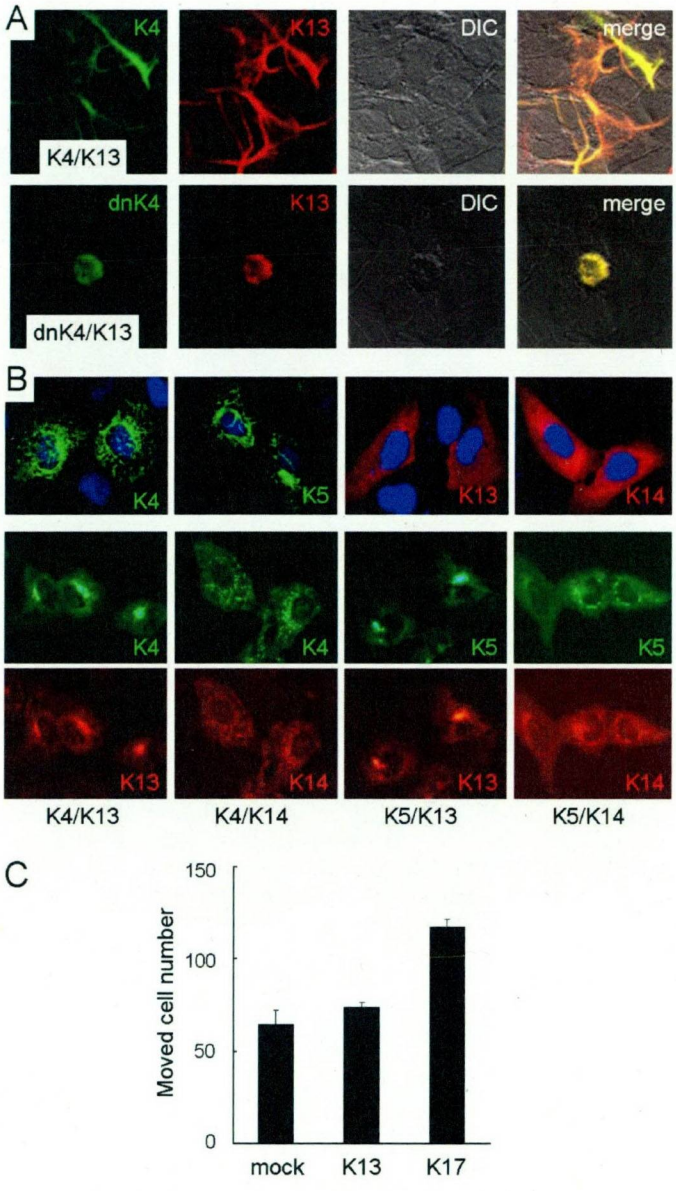

Figure 5. A) Ca9-22 cells cotransfected with K4 or dominant negative K4 (dnK4) and K13, tagged with GFP and RFP, respectively. B) K4/K14 or K5/K13 pairs can be incorporated into the cytoskeletal networks similar to K4/K13 and K5/K14 pairs. U2OS cells were transfected with GFP-K4, GFP-K5, K13-RFP and K14-RFP at the indicated combination. C) Cell movement assay. HEK293T cells were transfected with a mock, K13 or K17 plasmid and were placed in a cell culture insert (BD Falcon) of 8 μm pores coated with Type IV collagen. After 24 hours, the cells that moved out to the external plate were counted. The results are given as a mean value of a triplicate experiment.
258x448mm (96 x 96 DPI)

Human Pathology (in press)

Comprehensive keratin profiling reveals different histopathogenesis of keratocystic odontogenic tumor and orthokeratinized odontogenic cyst

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Keywords: Keratocystic odontogenic tumor; Orthokeratinized odontogenic cyst; Keratin; Gli2; K17

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Summary

Keratocystic odontogenic tumor is a cystic lesion which behaves more aggressively than other jaw cysts. One of its characteristic histological features is a parakeratinized uniform layer of lining epithelium. A jaw cyst lined with orthokeratinized epithelium is called an orthokeratinized odontogenic cyst. These keratinized jaw cysts are thought to be separate entities, although their histopathogenesis has not been fully assessed. To better understand these lesions, we performed comprehensive immunohistochemical profiling of the keratin expression of each.

Orthokeratinized odontogenic cysts expressed keratin 1, keratin 2, keratin 10 and loricrin, suggesting differentiation toward normal epidermis. Keratocystic odontogenic tumors expressed keratin 4, keratin 13, keratin 17 and keratin 19, which is a unique expression pattern reminiscent of a mucosal squamous epithelium and an epithelial appendage. In neonatal rat tooth germ, cells strongly positive for keratin 17 and keratin 19 were observed, specifically in the dental lamina, implying the origin of keratocystic odontogenic tumor. GLI2, a downstream effecter of hedgehog signaling, was significantly expressed in keratocystic odontogenic tumor and basal cell carcinoma, accompanied with robust expression of keratin 17, mTOR and BCL2. The expression of these GLI2- or keratin 17- related factors was not significantly observed in orthokeratinized odontogenic cysts. These findings provide evidence to support the viewpoint that keratocystic odontogenic tumor and orthokeratinized odontogenic cyst are separate entities, and furthermore suggest their characteristic histology, pathogenesis and biological behaviors.

1. Introduction

Keratocystic odontogenic tumor (KCOT, also known as odontogenic keratocyst) is an intraosseous lesion that accounts for about 10% of jaw cysts [1]. Approximately 5% of KCOT occurs as multiple lesions, and a few of those cases occur as a manifestation of nevoid basal cell carcinoma syndrome (NBCCS) [2]. KCOT shows a characteristic histological appearance. The cystic space is lined with a uniform parakeratinized squamous epithelium of 5-10 cell layers. The basal cells are aligned with vertically elongated nuclei. Mitotic activity is higher than other odontogenic cysts [3,4]. KCOT tends to recur after treatment, hence a correct diagnosis is essential [1,5]. Several lines of evidence suggest that constitutive activation of hedgehog (Hh) signaling is a major causative factor for the development of KCOT and basal cell carcinoma (BCC), even in solitary cases [6-8]. Because of these features, it has been proposed that this cystic lesion should be regarded as a benign neoplasm [1,5,9]. About 10% of keratinizing jaw cysts are lined predominantly by orthokeratinized epithelium, and this variant is called orthokeratinized odontogenic cyst (OOC) [10]. OOC exhibits a less aggressive behavior with a low recurrence rate [11]. It is generally accepted that OOC should be regarded as a separate entity from KCOT [9]. However, the diagnostic criteria are yet descriptive and the biological properties of these lesions have not been fully assessed. Therefore, the rationale for their separation, which is mainly based on the mode of keratinization, should be further evaluated.

Keratin is an epithelial-specific intermediate filament protein, whose production is the primary function of squamous cells. Keratin has many subtypes: the human genome contains 37 functional epithelial keratin genes. Among them, keratins from keratin 1 (K1) to K20 are major subtypes that are expressed in considerable amounts. These subtypes can be divided into basic (K1-K8) and acidic (K9-K20) ones; both types are coexpressed, dimerize together and form a cytoskeleton to maintain the cell shape and the integrity of the epithelium. The expression is regulated so that specific sets of keratins are produced depending on cell type and differentiation state. For example, a glandular epithelium typically expresses keratins of a low molecular weight such as K7, K8, K18, K19 and a squamous epithelium of skin express K1, K2 and K10 as well as Loricrin (LOR) whereas a squamous epithelium of mucosa expresses K4 and K13. Therefore, the evaluation of the keratin expression profile can facilitate cell typing and identification [12].

Since the property of a cell greatly depends on its lineage and differentiation state, keratin profiling of KCOT and OOC would provide essential information for understanding the pathogenesis and the biological behavior of these diseases. Although previous reports described expression of some individual keratins in these lesions [13-16], the comprehensive keratin profiles and their pathological significance are yet to be elucidated.

In this study, we examine the expression of all the major keratins in KCOT and OOC. The comparison of profiles reveals keratins

which characterize KCOT and OOC, and which explain their histology, further suggesting their pathogenesis and biological behaviors.

2. Materials and methods

2.1. Tissue specimens

Surgical specimens resected in the Hospital of Tokyo Medical and Dental University were collected. The pathological diagnoses were KCOT (20), OOC (20), dentigerous cyst (10), epidermoid cyst (10), ameloblastoma (7) and BCC (6). The clinical data of KCOT and OOC cases are summarized in Table 1. Six cases of KCOT occurred as multiple lesions. The tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. A neonatal rat (F344/Jcl) was sacrificed and was fixed in 4% paraformaldehyde/PBS for 2 days, decalcified in 10% EDTA for 1 week, dehydrated and embedded in paraffin. The experimental procedures were approved by the university ethics committee.

2.2. Immunohistochemical staining

Immunohistochemical staining was performed according to the standard protocol. For antigen retrieval, the sections were placed in TE buffer (10 mM Tris (pH = 9.0), 1 mM EDTA) and autoclaved at 120 °C for 20 min. The antibodies used in this study are listed in Table 2. The dilution factors for all primary antibodies were 1:500. The sensitivities and specificities of these antibodies were confirmed in a pilot experiment using specimens of normal skin, tongue, salivary gland and digestive tract as a reference tissue. For incubation with anti-Gli2 antibody, Can Get Signal (Toyobo, Osaka, Japan) was used. EnVision Dual Link (Dako, Glostrup, Denmark) or ImmPRESS anti-Goat Ig Kit (Vector Laboratories, CA, USA) was used as the secondary antibody.

3. Results

3.1. Keratin profiles of KCOT and OOC are distinctive

The immunohistochemical staining results of representative cases of KCOT and OOC are shown in Fig. 1. The expression was evaluated at the site without inflammation to avoid misinterpretation due to reactive changes. The keratin profiles of KCOT and OOC are summarized and depicted by a schematic illustration (Fig. 2). The most relevant keratins for distinguishing KCOT from OOC were K4, K13, K17 and K19, which were consistently and strongly positive in KCOT and almost negative in OOC; and K1, K10 and LOR, which were consistently positive in OOC and negative in KCOT. In KCOT, K17 and K19 were expressed both in the basal and suprabasal cells, whereas K4 and K13 were expressed in the superficial layer. In OOC, K1 and K10 were expressed in the suprabasal cells, and LOR was expressed superficially. Expression of several keratin subtypes exhibited case-dependent variations but no apparent subgroup defined by the keratin profile was noted. No significant difference was observed in the keratin profiles between solitary KCOT and multiple KCOT (data not shown). These results indicated that KCOT and OOC expressed unique sets of keratin subtypes, suggesting that each constitutes a distinct entity defined by the keratin profile.

3.2. The keratin profile of OOC is almost identical to that of the epidermis

For better understanding the origin and the biological properties of a tumor, it is informative to identify the normal tissue, whose differentiation state resembles that of the tumor cells. We searched for a tissue that exhibited the keratin profile similar to KCOT and OOC. The keratin profile of OOC was almost identical to that of epidermis, whose basal cells were positive for K5, K14, K15 and whose suprabasal cells were strongly positive for K1, K2, K10 with superficial LOR expression (data not shown). K4(+)K13(+) phenotype was seen in the non-keratinized squamous epithelium such as that of oral or esophageal mucosa, and K17(+)K19(+) phenotype was seen in epithelial appendages such as hair follicles, but the K4(+)K13(+)K17(+)K19(+) phenotype of KCOT was unique and was different from any normal adult tissues.

3.3. The keratin profile of KCOT is similar to that of the dental lamina

We extended the examination of keratin expression to neonatal oral tissue including tooth germ. Because of the difficulty of obtaining a human tissue, a neonatal rat was used. In the neonatal tooth germ, the cells with distinct K17(+)K19(+) phenotype were observed in the dental lamina, whereas the other part of the tooth germ and the oral epithelium expressed only a trace of these keratins (Fig. 3, Table 2).

3.4. Keratin profiles of other cystic lesions

We examined the keratin profile in other cystic lesions that are considered in the differential diagnosis of KCOT and OOC. Data for the relevant keratin expressions are summarized in Table 2. The keratin profile of dentigerous cyst was different from that of KCOT in its absence of K17 expression (Supplementary figure S2). Cystic type ameloblastoma showed a keratin profile similar to that of KCOT, but the expression of K17 was superficial, contrasting with the diffuse expression in KCOT (Table3, Supplementary figure S2). Epidermoid cyst showed an almost identical keratin profile to OOC (Supplementary figure S2).

3.5. Expression of GLI2 and mTOR is associated with K17 expression in KCOT

Since these results suggested the significance of K17 in the pathogenesis of these lesions, we examined the expression of K17-related factors in KCOT, OOC and BCC. GLI2, a downstream effecter of Hh signaling and an inducer of K17 expression, was significantly upregulated in the tumor cells of all 6 BCC cases, and was strongly expressed in all cases of KCOT, exhibiting nuclear localization (Fig. 4). Keratins considerably expressed in BCC were only K17 as well as basal cell keratins K5, K14 and K15, and thus K17 was consistently co-expressed with GLI2 both in BCC and KCOT. Mammalian target of rapamycin (mTOR), which is an essential regulator of cell growth and is activated by K17 expression, was robustly expressed in BCC and KCOT. In contrast to KCOT, which showed diffuse strong expression of mTOR, the expression in OOC was largely restricted to the basal cells (Fig. 4). Anti-apoptotic protein BCL2 was also strongly expressed in BCC and KCOT. OOC was almost negative for GLI2 and BCL2, as well as for K17.

4. Discussion

K4 and K13 are differentiation-related keratin pairs that are predominantly

expressed only in the suprabasal layer of non-keratinized squamous epithelium (Supplementary figure S3). The superficial cells strongly positive for K4 and K13 in KCOT were mostly parakeratinized; therefore, their expression in KCOT appeared to associate with parakeratinization characteristically observed in this lesion. Similarly, K1 and K10 are specific for the suprabasal cells of keratinized squamous epithelium, and appeared to associate with orthokeratinization in OOC. Typically, the expressions of the K4/K13 pair and the K1/K10 pair are mutually exclusive of each other, and the mode of keratinization, which is one of the major histological features separating KCOT and OOC, is thus due to the selection of these two pairs of keratin subtypes. OOC also expressed K2 and LOR, which are expressed in the upper spinous cell layer and in the cornified envelope, respectively. These results indicated that the differentiation of OOC cells as epidermis is almost complete, which may associate with the unaggressive behavior of OOC. In contrast, KCOT showed a unique and unusual keratin profile, suggesting dysregulation of differentiation.

K8 and K18 are the most primitive forms among keratin families, and their expression starts as early as in ectoderm [12]; both are typically expressed in a simple epithelium, but not in a squamous epithelium. Occasional expression of K8 and K18 in KCOT suggests that the lesion may arise from cells that retain primitive properties, as is seen in embryonic epithelium. In a single-layered simple epithelium, keratin filaments (composed of K8, K18 and K19) distribute with apical polarization, contrasting with a squamous epithelium whose keratin filaments show baso-apically uniform distribution. The significance of the asymmetric distribution of keratin filaments is yet poorly understood, but it has been suggested that these keratins participate in the generation of epithelial cell polarity [17]. Abundant expression of K19 may associate with generation of a polarized, vertically-elongated cell shape observed in KCOT.

K6, K16 and K17 are related to regeneration and they are induced in a hyperproliferative epithelium after injury [12,18]. Among these, K17 was consistently and strongly expressed in KCOT. K17 increases mTOR signaling activity, stimulating protein synthesis and cell growth [19]. Robust expression of K17 and concomitant upregulation of mTOR in KCOT may associate with its proliferative and aggressive behavior.

Strong expression of K17 and K19 was distinct from normal squamous epithelium and seemed a unique feature of KCOT. Interestingly, cells strongly positive for K17 and K19 were observed exclusively in a dental lamina of a late-stage tooth germ. This seemed to support the notion that KCOT arises from the remnant of dental lamina cells.

A striking similarity of the keratin profile was observed also between KCOT and the hair follicle bulge, which expressed both K17 and K19. Although the bulge becomes to express various hair keratins instead of K4 and K13, the keratin profile of the bulge cells is identical to the basal cells

of KCOT, which are positive for K5, K14, K15, K17 and K19. Considering the analogous histogenesis between tooth germ and hair follicle development, this similarity provides an implication for the pathogenesis of KCOT.

NBCCS is an autosomal dominant genetic disease that exhibits multiple KCOT and BCC. NBCCS is typically caused by a mutation in the hedgehog (Hh) receptor *PATCHED* gene, which leads to constitutive active Hh signaling [8]. BCC is thought to arise from pluripotent hair follicle cells [20], and the Hh signaling is essential not only for the occurrence of BCC but also for hair follicle development [21]. Aberrant Hh signaling may also associate with non-syndromic KCOT and BCC [7,8], and in fact, we demonstrated that the effecter of Hh signaling GLI2 was significantly expressed both in KCOT and BCC, accompanied with upregulation of K17 that is a putative downstream target of Hh signaling [22]. These results suggest that GLI2 upregulation, either or not by aberrant Hh signaling, is one of the direct causes of KCOT development.

K5-Gli2 transgenic mice develop jaw cysts derived from epithelial rests of Malassez [23]. These cysts show histological features of OOC, but not of KCOT. This animal experiment implied that GLI2 is important also for OOC development. However, GLI2 expression was not detected in human OOC, leaving the origin and pathogenesis of OOC yet to be elucidated.

We found that the anti-apoptotic protein, BCL2 was upregulated in KCOT. Since BCL2 has been shown to be a direct transcriptional target of GLI2 [24], the GLI2-BCL2 cascade may play a role in KCOT development, possibly by impaired apoptosis to correct secondary mutation events.

In conclusion, keratin profiling indicates that the differentiation states of KCOT and OOC are distinct and completely different from each other. The repertoire of the expressed keratin subtypes seems to associate with their characteristic histology and further suggests their pathogenesis and biological behaviors.

Acknowledgements

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Table1
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	age/sex	size	location		age/sex	size	location
KCOT1	29/F	30×30	mandible	OOC1	21/F	NA	mandible
KCOT2	14/F	15×21	mandible	OOC2	22/M	25×32	mandible
KCOT3	15/F	16×12	mandible	OOC3	22/M	30×35	mandible
KCOT4	19/M	NA	mandible	OOC4	26/F	NA	mandible
KCOT5	21/M	27×21	maxilla	OOC5	27/F	40×40	maxilla
KCOT6	34/F	36×30	mandible	OOC6	27/F	NA	mandible
KCOT7	51/F	NA	maxilla	OOC7	28/F	18×17	mandible
KCOT8	56/M	12×20	mandible	OOC8	28/M	30×15	mandible
KCOT9	21/F	15×20	mandible	OOC9	28/F	25×30	maxilla
KCOT10	59/M	19×10	mandible	OOC10	29/F	NA	mandible
KCOT11	65/M	NA	mandible	OOC11	35/M	NA	mandible
KCOT12	65/M	13×12	mandible	OOC12	39/M	30×25	mandible
KCOT13	72/M	NA	mandible	OOC13	40/M	34×20	mandible
KCOT14	19/F	21×16	mandible	OOC14	41/M	40×30	maxilla
KCOT15	33/M	max 45×15	multiple(3)	OOC15	41/M	NA	maxilla
KCOT16	10/M	max 27×29	multiple(3)	OOC16	51/M	NA	mandible
KCOT17	13/F	max 30×22	multiple(2)	OOC17	60/M	NA	mandible
KCOT18	17/F	NA	multiple(5)	OOC18	64/M	NA	mandible
KCOT19	13/F	NA	multiple(4)	OOC19	68/M	30×20	mandible
KCOT20	11/F	max 37×33	multiple(5)	OOC20	11/M	50×25	maxilla

Table2
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Antigen	Supplier	Clone name
K1	Santa Cruz	N-20
K2	Progen	Ks2.342.7.1
K4	Epitomics	EP1599Y
K5	Monosan	XM26
K6	Neomarkers	LHK6B
K7	DAKO	RN7
K8	Novacastra	TS1
K9	EuroDiagnostica	Ks9.70/Ks9.216
K10	Neomarkers	DE-K10
K13	Novacastra	KS-1A3
K14	Abcam	LL002
K15	Epitomics	EPR1614Y
K16	Neomarkers	LL025
K17	Dako	E3
K18	Dako	DC10
K19	Epitomics	EPR1579Y
K20	Dako	PW1
Hair Keratin	Santa Cruz	AE13
LORICRIN	Covance	PRB-145P
BCL2	Dako	clone124
GLI2	Santa Cruz	H-300
mTOR/FRAP	Epitomics	Y391

Table3
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	K17	K19
KCOT	+ +	+ +
OOC	-	-
inner ameloblast	-	-
outer ameloblast	+	-
stellate reticulum	-	-
dental lamina	+ +	+ +
dentigerous cyst	-	+ +
cystic ameloblastoma	+	+ +

Figure1
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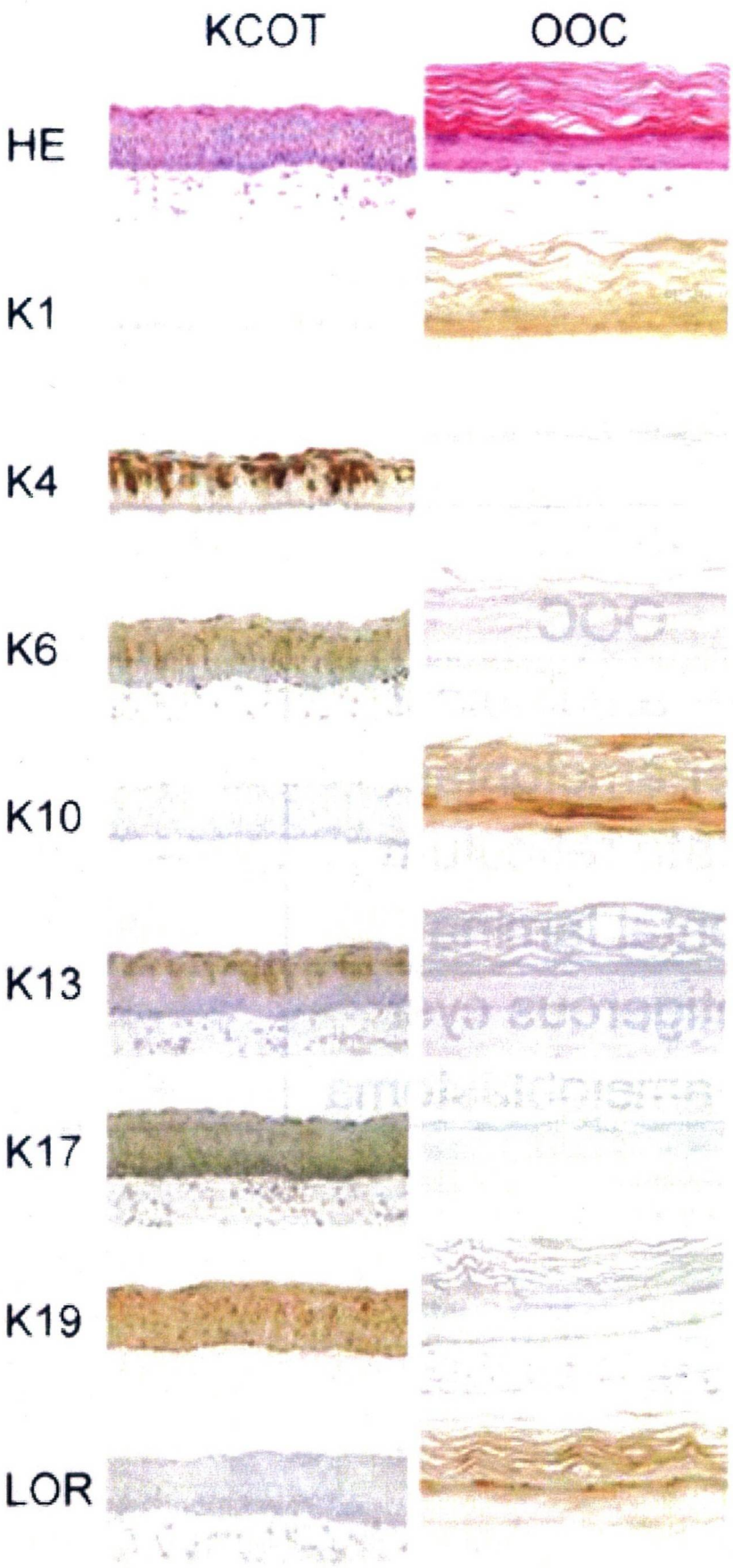

Figure2
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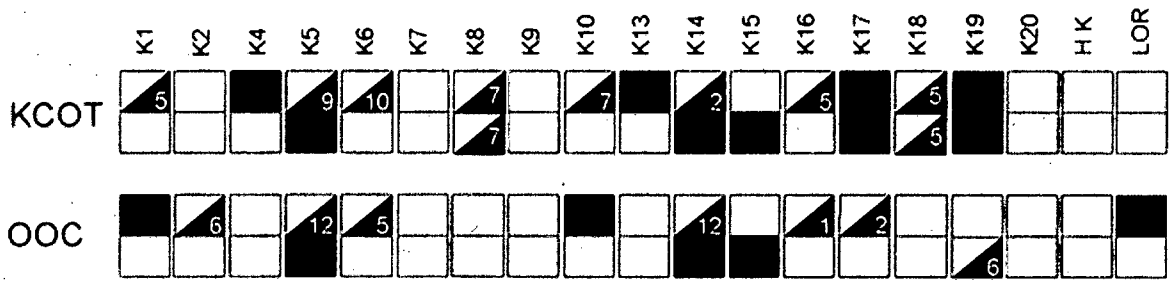

Figure3
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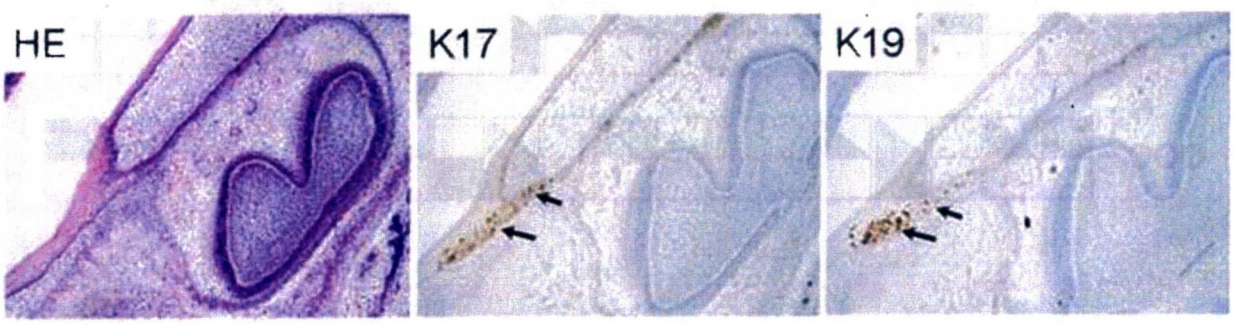

Figure4
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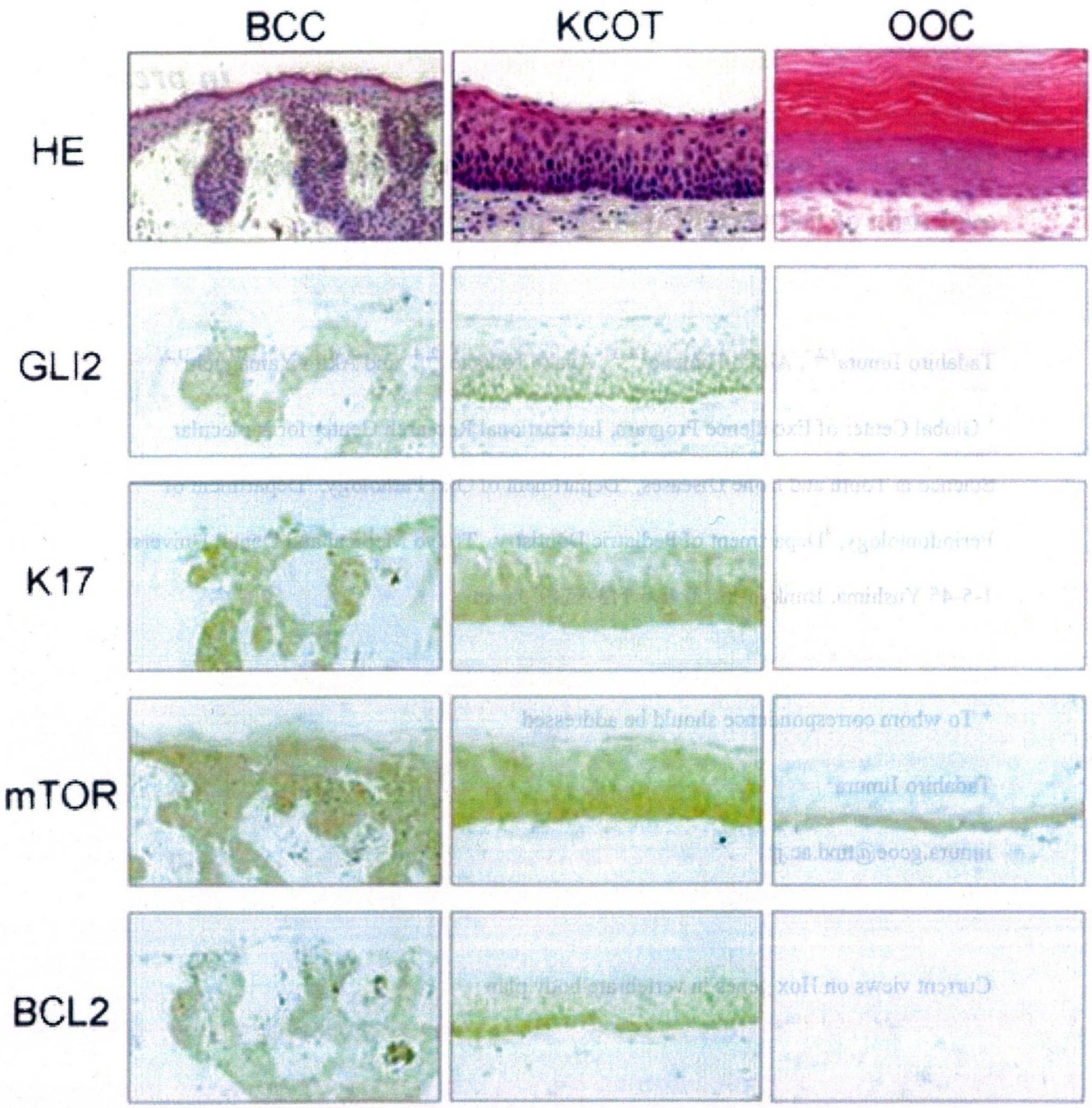

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Hox genes, a molecular constraint for the development and evolution of the vertebrate body plan

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Current views on Hox genes in vertebrate body plan

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