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ORIGINAL ARTICLE

Altered expression of cell–cell adhesion molecules β-catenin/E-cadherin and related Wnt-signaling pathway in sporadic and syndromal keratocystic odontogenic tumors

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Abstract Differential diagnosis of the keratocystic odontogenic tumor (KCOT) still represents a challenging problem especially if compared with the dentigerous cyst, which is similar in clinical and radiological course. Histological assessment of this entity may therefore draw crucial attention since various radical procedures are recommended for such lesions in contrast to dentigerous cysts. Since recent reports could prove the involvement of wingless (Wnt)-signaling pathway and β-catenin in the pathogenesis of many odontogenic and neoplastic lesions indicating impairment of cell–cell adhesion, we investigated the expression of two Wnt-signaling pathways, Wnt-1 and Wnt-10A as well as β-catenin and E-cadherin along with other related proteins in both lesions. We found a significant down-regulation in the expression of cell adhesion proteins β-catenin and E-cadherin along with alteration of Wnt-1 and Wnt-10A expression in the epithelium of KCOT. We assessed a specific focal distribution pattern of p63 in the suprabasal cell layer and a significant up-regulation of cyclin D1. Furthermore, laminin α-2 was a characteristic marker labelling only the basement membrane of dentigerous cysts. These results provide a new hypothesis explaining a molecular mechanism to understand initiating and development of KCOTs and an alternative therapeutic approach, especially for syndromal patients, where these multilocal lesions may involve and destroy wide orofacial bony structures.

Keywords KCOT · Wnt-1 · Wnt-10A · β-catenin · E-cadherin · P63 · CD1

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Introduction

Keratocystic odontogenic tumor (KCOT) has recently been suggested to describe the lesion previously named odontogenic keratocyst. Clinical and immunohistological research discussed and queried the cystic nature of this lesion, since clinical course showing recurrence and local aggressive invasion suggests rather a neoplastic entity [1]. Histological assessment of this entity may therefore draw crucial attention, since various radical procedures are recommended for such lesions in contrast to dentigerous cysts [2]. In order to identify relevant pathognomonic features of occasionally occurring sporadic as well as inherited variant, usually described as syndromal KCOT, immunohistochem-

ical expression of extracellular matrix proteins (ECM) has been assessed showing however no specific differences [3, 4]. Recent reports on the important role of two wingless genes (Wnt and Wnt-10A) during normal tooth development aroused interest to investigate this signal pathway in neoplastic odontogenic lesions [5–7].

Based on the hypothesis that KCOT may represent dysregulation of Wnt pathway on one or more levels including its intracellular transcription, we investigated this specific transduction pathway along with its main downstream protein β-catenin and related cell–cell adhesion molecule E-cadherin. Furthermore, we studied the related cell cycle proteins p53, p63, Ki-67, and cyclin D1 and chose the morphological immunohistological approach to reveal subcellular localization of Wnt-1 and Wnt-10A in the epithelium of KCOTs and dentigerous cysts. Supplemental investigations were also performed to evaluate expression of the extracellular matrix proteins (ECM) tenascin and laminin α-2 in order to find out potential pathognomonic features, which may characterize the keratocystic odontogenic tumor.

Materials and methods

Study design

Specimens were collected from two cooperating university hospitals in Germany and amounted to 18 KCOT and 8 dentigerous cysts. KCOTs were derived from primary and recurrent lesions from 4 patients with nevoid basal cell carcinoma syndrome (Gorlin–Goltz-syndrome) (6 specimens) and 12 sporadic cysts of non-syndromal patients. Investigations were performed after approval of local authority and have been conducted according to Declaration of Helsinki principles.

Biopsies were fixed in neutral phosphate buffered 4% formalin. After a minimum of 48 h of fixation, the tissue was trimmed and processed by standard paraffin-embedding methods. Sections were cut at 4 μm, deparaffinized, and then stained. For immunohistochemical staining, the Alkaline Phosphatase Anti Alkaline Phosphatase (APAAP) technique was used to visualize the primary antibodies, namely monoclonal mouse anti-human tenascin, Ki-67, p53, p63, cyclin D1, LAMA2, Wnt-1, Wnt-10A, β-catenin, and E-cadherin (Table 1). The APAAP technique was used for visualization of the bound primary antibodies. The secondary rabbit anti-mouse antibody and the APAAP complex were diluted 1:50 (both from DakoCytomation, Denmark). Naphtol-AS-biphosphate (Sigma, USA) and new fuchsin (Merck, Germany) were respectively used as substrate and developer. As negative control, the primary antibody was replaced by a non-immune serum.

Evaluation of immunostaining

Expression of cyclin D1 and p53 was evaluated as previously described [8]. Positive stained cells within the epithelium were counted using a grid raster in ten randomized view fields and expressed as a percentage of positive cells of the total number of investigated epithelial cells. Considering the hot spots in the view field, Ki-67 positive cells were evaluated per 300 epithelial cells and expressed as mean±SEM.

In order to evaluate the degree of immunostaining of tenascin-C, β-catenin, E-cadherin, Wnt-1, Wnt-10A, p63, as well as laminin α-2, a semi-quantitative score was introduced. Ten view fields of each sample were chosen at random, and the area of positive reaction in relation to the whole view field was calculated using the Soft Imaging System analysis®. The score was defined as follows: staining of 0–25% of view field, score=+; staining of 25–50% of view field, score=++; staining of 50–75% of view field, score=+++; staining of more than 75% of view field, score=++++.

Results

Depending on potential target site of cell disintegrity in the studied lesion, following cellular levels were investigated: cystic epithelium, basal membrane, and adjacent stroma.

Epithelial side

β-catenin

Regular membranous staining was detected in the epithelium of dentigerous cysts labelling the inner surface of cell membrane of all layers from the basement membrane to the cystic lumen (Fig. 1g). The crucial difference seen in sporadic and syndromal KCOTs was the down-regulation of β-catenin expression, especially in the basal and luminal parakeratinized cell layers (Fig. 1h, i). Loss of β-catenin staining was significantly higher in syndromal than in sporadic KCOTs.

E-cadherin

Regarding dentigerous cysts, a similar regular distribution pattern and epithelial cell membrane staining could be described for E-cadherin, which plays the extracellular role in the cell–cell-adhesion (Fig. 1j). In contrast, KCOTs showed a moderate to high-grade loss of E-cadherin staining in all samples (Fig. 1k, l), especially in syndromal lesions, which displayed more impaired staining. The

Table 1 Antibodies used for the immunohistological investigations and their related data

Antibody	Clone	Dilution	Source
Wnt-1	Not provided	1:500	Assay Designs, USA
Wnt-10A	Not provided	1:200	Novus Biologicals, USA
β-catenin	17C2	1:200	Novocastra, UK
E-cadherin	4A2C7	1:75	Zytomed, Germany
Ki-67	MM1	1:100	Novocastra, UK
p53	Bp 53-12	1:1,000	Santa Cruz Biotechnology, USA
p63	4A4	1:200	LABVison, UK
Cyclin D1	SP4	1:50	LABVision, UK
Laminin α-2	2D4	1:50	Biozol Diagnostica, Germany
Tenascin-C	BC4	1:300	Dr. L. Zardi, University of Genoa, Italy

region of lost reaction was frequently located in the basal cell layer or at the luminal side.

p63, p53, Ki-67, and CD1

Positive reaction for p53 and Ki-67 was detected in all samples of the three entities. Although only scattered epithelial cells of dentigerous cysts displayed positivity to p53 and Ki-67, while a more intense reaction was observed in KCOTs, we could not asses any significant differences of immunostaining among the lesions (p53/Ki-67=10±2%/2–3% of epithelial layer in dentigerous cysts; 8±2%/1–3% of epithelial layer in sporadic KCOTs and 6±1%/1–3% of epithelial layer in syndromal KCOTs, respectively (Table 2).

While epithelial cell nuclei of dentigerous cysts showed ubiquitary staining with p63, epithelial layer of KCOTs derived from sporadic and syndromal patients displayed a totally different pattern. Only basal and suprabasal cell layers displayed a nuclear expression indicating activation of p63; however, pathognomonic multifocal labelling of proliferating suprabasal cell layer was evident in all samples (Figs. 1m–o and 3).

Scattered faint nuclear labelling with cyclin D1 was seen in the epithelial layer of dentigerous cysts counting for 4±1% of investigated view fields. A significant increase in this rate was assessed in syndromal and to a lesser extent in sporadic KCOTs (38±8% and 33±6%, respectively).

Wnt-1 and Wnt-10A

Samples derived from dentigerous cysts did not show any immunostaining with Wnt-1 or Wnt-10A (Fig. 1a–f). In contrast, we observed two different pathognomonic expression patterns of Wnt-1and Wnt-10A in sporadic and syndromal KCOTs. On one side, all cells of the epithelial layer displayed an intense nuclear labelling of Wnt-1, on the other side, cytoplasmic expression of Wnt-10A was detected in the suprabasal epithelial layer of KCOT (Fig. 1e, f). No significant difference was assessed between syndromal and sporadic types of KCOTs.

Basement membrane and stroma

Laminin α-2 (merosin)

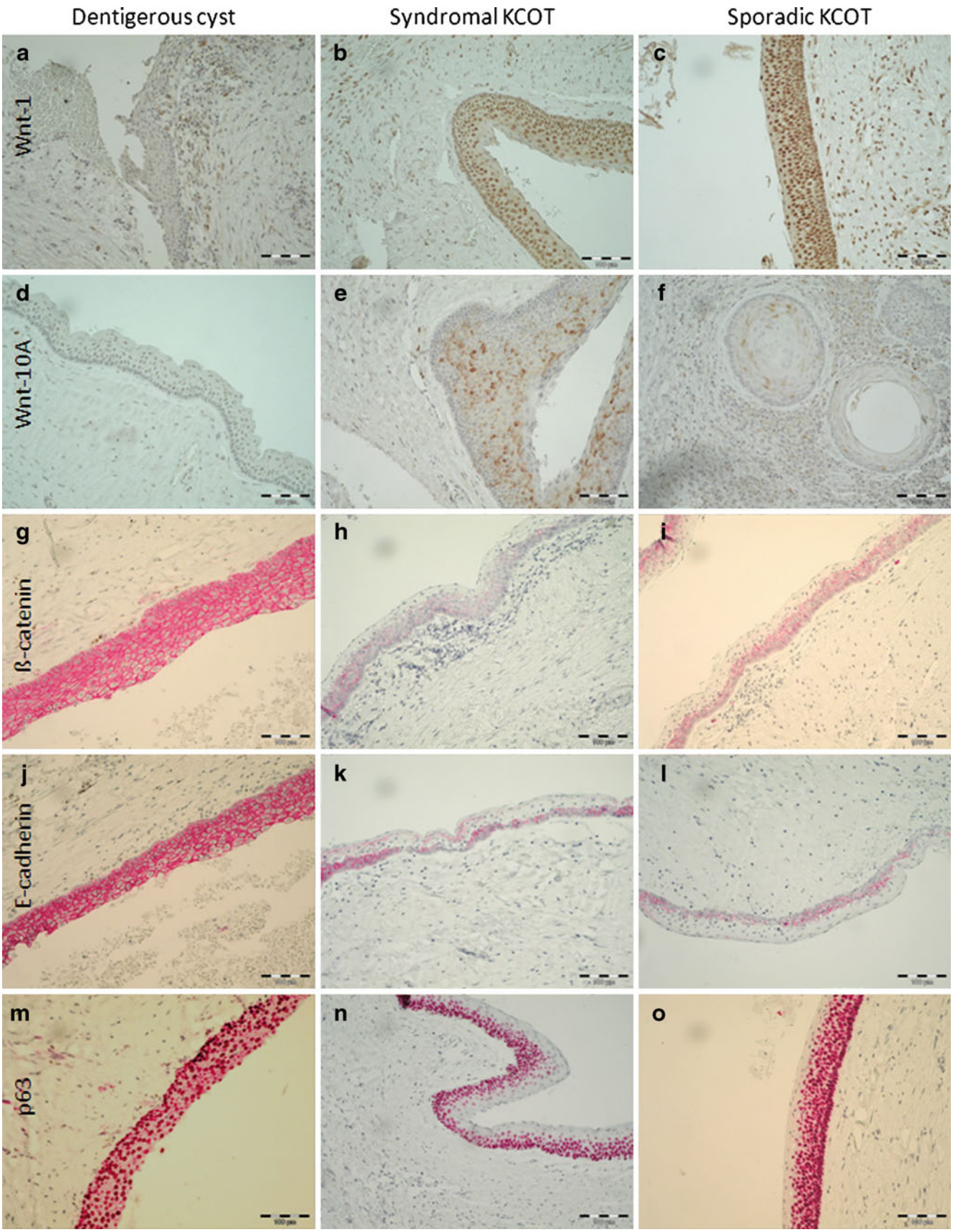
Laminin-2 (LN-2, α2β1γ1) positive labelling could be detected as an irregular linear staining labelling the basement membrane of the epithelium in dentigerous cysts (Fig. 2a). This expression was also evident in the basal membrane around epithelial cross sections of dermal papillae in the stroma (Fig. 2b). Neither sporadic nor syndromal KCOTs showed any expression (Table 2).

Tenascin-C

Tenascin-C was expressed as a fibroreticular band at the dermal–epidermal junction, staining the stromal border of the basement membrane intensively while becoming weaker away from this border. Such a reaction was variable displaying interruptions along the basal membrane in all specimens of sporadic and syndromal KCOT as well as in dentigerous cysts. However, KCOTs always showed a significantly more intense staining in contrast to dentigerous cysts (Table 2).

Discussion

Keratocystic odontogenic tumors develop sporadically and in syndromal patients with nevoid basal cell carcinoma syndrome or Ehlers–Danlos syndrome [9–11]. Evidence has been shown that pathogenesis of both lesions involves a process by which the tumor suppressor gene Patched (PTCH1) is mutated and inactivated leading to failed negative feedback control of the Sonic hedgehog pathway (SHH). The result is dysregulation of the oncoproteins


Dentigerous cyst
Syndromal KCOT
Sporadic KCOT
a
b
c
Wnt-1
d
e
f
Wnt-10A
g
h
i
ß-catenin
j
k
l
E-cadherin
m
n
o
p63

◀ **Fig. 1** Immunostaining of a dentigerous cyst (**a**) using Wnt-1 shows no labelling in the epithelial cell layer, while KCOTs derived from syndromal patients (**b**) as well as sporadic ones (**c**) displayed a strong regular nuclear staining with Wnt-1. No Wnt-10A staining is evident in dentigerous cysts (**d**). In contrast, suprabasal epithelial layer in syndromal and sporadic KCOTs displayed intracellular immunostaining (**e** and **f**, respectively). Immunohistological expression of β-catenin (**g**) and E-cadherin (**j**) in dentigerous cysts showing a regular comprehensive labelling with both antibodies throughout the epithelium including the basal cells as well as luminal cell layers. KCOTs of both syndromal and sporadic origin (**h**, **k**, **i**, and **l**, respectively) displayed a faint staining especially in the suprabasal layer, whereas no immunostaining was observed in basal cells and superficial cell layers on the luminal side. p63 labelling of dentigerous cysts (**m**) showing homogenous nuclear staining of all epithelial cell layers. Loss of p63 staining in the superficial cell layers in both syndromal (**n**) and sporadic (**o**) KCOTs along with increased proliferation of p63 positive suprabasal cells

cyclin D1 and p53 [12–14]. Based on this hypothesis, recent studies investigated and assessed an aberrant activation of SHH signaling and expression of related target genes in KCOTs [15–17]. Furthermore, potential activation of the Wnt pathway is also associated with up-regulation of the down-stream genes in the hedgehog/patched pathway [18, 19], and linkage between SHH and Wnt pathways has also been assessed during craniofacial development [20]. Nevertheless, little is known about the role of Wnt signaling in syndromal and sporadic KCOTs, particularly regarding the related down-stream protein and regulator β-catenin and its adhesion co-element E-cadherin.

This study is the first to address the Wnt signaling and related adhesion proteins in dentigerous cysts and KCOTs. The results may therefore be understood and interpreted in the light of previous knowledge gained from similar experimental works.

In mammals, Wnt-1 acts as a growth factor that is secreted and becomes associated with cell surface [21, 22]. It modulates cell–cell adhesion by stabilizing the β-catenin binding to the cell adhesion protein cadherin [23]. Since β-catenin regulates together with α-catenin the cadherin function, aberrant expression of β-catenin and cadherin leads in turn to decreased cell–cell adhesion and disruption of tissue morphogenesis [24], which is correlated with neoplastic cell invasion [25].

We assessed a significant decrease in the membranous expression of β-catenin and E-cadherin in all samples of sporadic and syndromal KCOTs, while dentigerous cysts showed, in contrast, regular staining from the basement membrane of the epithelial layer up to the luminal surface (Fig. 1g, j).

The cadherin-mediated cell adhesion acts as an invasion suppressor system in cancer cells, since non-invasive cells can be transformed into invasive ones when cadherin function is blocked by related antibodies [26, 27]. In our study, we showed a significant down-regulation of E-cadherin in KCOTs, which explains the invasive growth of KCOT. In contrast to dentigerous cysts, disruption of cell–cell adhesion molecules was associated with strong nuclear staining with Wnt-1 throughout the epithelium of KCOTs. These results are in accordance with previous studies reporting overexpression of Wnt-1 in gastric and pancreatic [28] as well as colorectal [29] and breast cancer cells [30], which detected a strong Wnt-1 staining in low grade tumors with similar local growth pattern like KCOTs.

Furthermore, a specific distribution pattern of Wnt-10A in both entities of KCOTs indicated a crucial difference to the dentigerous cysts, which displayed no expression of Wnt-10A. The crucial role of Wnt-10A in the embryologic development and morphogenesis of tooth epithelium has been proven in the sense of up-regulation [31, 32].

Whether this up-regulation of Wnt-1 and Wnt-10A expression represents a reply on the decreasing β-catenin/E-

Table 2 Immunohistological evaluation of investigated dentigerous cysts as well as sporadic and syndromal keratinized cystic odontogenic tumors

Immunostaining	Dentigerous cysts	Sporadic KCOTs	Syndromal KCOTs
Wnt-1*	(+)	++++	++++
Wnt-10A*	0(+)	+++	+++
β-catenin*	++++	++	(+)
E-cadherin*	++++	++	+(+)
Ki-67	2–3%	1–3%	1–4%
p53	10±2	8±2	6±1
p63*	++++	++ (Focal proliferation)	++ (Focal proliferation)
Cyclin D1*	4±1%	38±8%	33±6%
Laminin α-2* (basement membrane)	+	0	0
Tenascin-C (epithelial–stromal interface)	++	++(+)	++(+)

*$p < 0.05$, statistically significant results

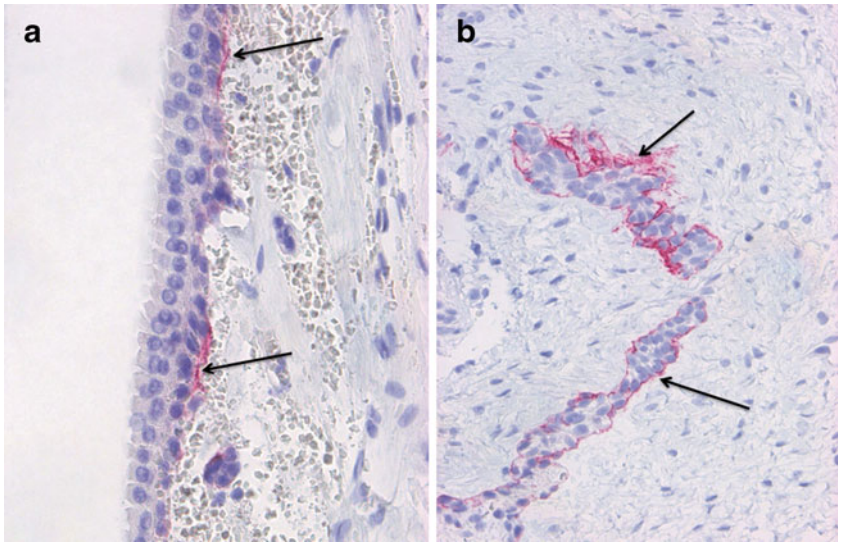


Fig. 2 **a** Immunostaining of dentigerous cysts with laminin α-2 displaying a regular linear labelling of the basement membrane (*arrows*). **b** Scattered distribution of epithelial islands sections (*arrows*) show the same immune expression patterns of laminin α-2

cadherin levels as an intact feedback regulation or impaired Wnt-signaling failed to induce regular down-stream proteins, could not be sufficiently explained in this study.

During tooth formation, the ECM plays a crucial role in the differentiation and development process. Once this is completed, a reactivation of epithelial remnants may initiate regrowth and development of odontogenic tumors [33, 34], which is associated with de-novo expression of ECM proteins like fibronectin, laminin, and tenascin.

We compared dentigerous cysts with KCOTs in regard to ECM expression. Tenascin-C and laminin α-2 expression was observed in both lesions, albeit in a different intensity pattern. We could particularly demonstrate an up-regulation of tenascin-C expression in the adjacent stroma of the epithelium of KCOTs. In this regard, our results are in accordance with the most representative studies of de Oliveira et al. and Amorim et al. who assessed similar findings, postulating that the aggressive nature and recurrence of KCOTs is associated with abnormal expression of tenascin [3, 4].

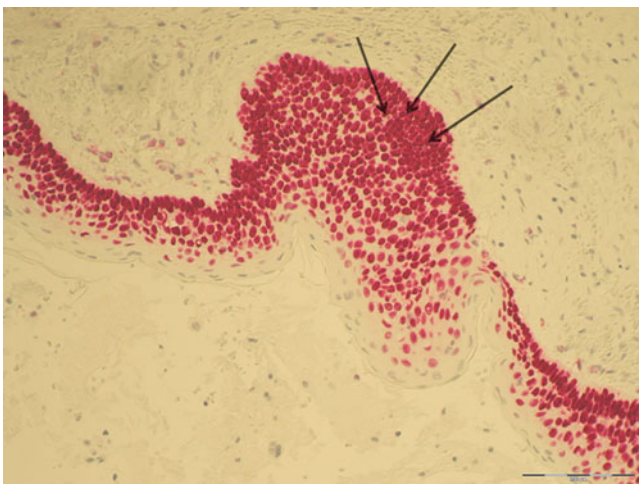


Fig. 3 Immunohistological investigation using p63 displaying multifocal pathognomonic strong labelling of increased proliferation of the suprabasal cell layer, which was evident in all samples of KCOTs

Participation of laminin α-2 in the Erk-related pathway, including activation of cyclin D1 and cell proliferation, has been proven on conjunctival epithelial cell line [35]. Similar results regarding odontoblast differentiation have also demonstrated the regulatory role of laminin α-2 in the expression of dentin sialoprotein in developing mice [36]. Therefore, the fact that only dentigerous cysts displayed intact regular expression of laminin α-2, but none of the KCOTs, emphasizes the particular role of laminin α-2 in the maintenance of basement membrane of normal cell–cell interaction in the odontogenic epithelium. A recently published study demonstrated similar results, indicating the relevance of laminin in the integrity of the basement membrane in verrucous carcinoma of the oral mucosa [37].

The p53 homologue p63 participates in cell proliferation and may also act as an oncogen [38–40]. Mutation of p63 implicates dramatic disorders, including absent hind limbs and craniofacial malformations, ectodermal dysplasia as well as acro-dermato-ungual-lacrimal-tooth-syndrome [39, 41].

As far as we know, only three studies investigated the expression of p63 in KCOTs and dentigerous cysts. Lo Muzio et al. observed moderate positivity throughout the epithelium of dentigerous cysts, while KCOTs displayed the most intense labelling especially in the basal and suprabasal layers. They postulated that p63 expression may be useful to identify lesions with aggressive and invasive proliferative activity. Foschini, in contrast, reported a more homogenous labelling pattern of p63 in the basal cells in primary KCOT but more comprehensive reaction in recurrent lesions. In the most recent study of Gurgel et al., increased cell proliferation and raised expression rate of p63 in both lesions was assessed [42].

In the present study, we found a different distribution pattern of p63 in the three entities. Basal and suprabasal cell layers of KCOTs displayed strong nuclear expression indicating activation of p63; however, the most distinct feature may represent the pathognomonic multifocal proliferation of the suprabasal cell layer (Fig. 3). This increased cell proliferation explains the infiltrative growth pattern and associated bone invasion of KCOTs. This interpretation is in accordance with the results of Foschini et al., albeit overexpression has mostly been shown in recurrent KCOTs [43].

In contrast to previous studies using a special proliferation antibody (IPO-38) [44], we could not observe any significant differences in the expression of Ki-67 and p53 among sporadic and syndromal KCOTs or dentigerous cysts indicating comparable cell proliferating rates. This discrepancy could be attributed to the fact that these studies investigated the differences between primary and recurrent KCOTs [42] or between sporadic KCOTs and those associated with Gorlin–Golz syndrome [45], but they did not consider dentigerous cysts as a comparable cystic lesion. Complementary data was provided by Slootweg, who showed a similar expression of Ki-67 and p53 in dentigerous and radical cysts [8, 46, 47]. Taking our results together with those of previous investigations, we suggest a similar biologic behavior of the two KCOTs entities concerning Ki-67 and p53 expression.

The Wnt target gene and cell cycle protein cyclin D1 was found to be up-regulated in the epithelium of KCOTs, particularly in the suprabasal layer. This may represent together with the overexpression of p63, a further indication of impaired cell cycle and in KCOTs. A recent study published by our group demonstrated a similar cyclin D1 expression in recurrent ameloblastoma, which is also in accordance with the report of Lo Muzio and Opitz, who postulated the close relationship between increased expression of the cell cycle protein cyclin D1 and cell invasion [45, 48–50].

Considering the results presented, a new pathogenetic approach can be postulated to understand the possible role of Wnt-signaling pathway and subsequent alteration of cell–cell adhesion in the development of the keratocystic odontogenic tumor. This may improve differential diagnosis and yield prerequisites for innovative therapeutic approach of this lesion.

Conflict of Interest None declared.

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