Abstract: Odontogenic tumors are a group of lesions arising from the odontogenic apparatus. Although the mechanism of oncogenesis and tumor progression in these lesions remains unknown, certain proteins, such as those involved in apoptosis, seem to be involved in the differentiation and proliferation of odontogenic epithelial cells. The aim of this study was to analyze the expression of p73 and TNF-related apoptosis-inducing ligand (TRAIL) in odontogenic tumors and cysts, and to clarify changes in the expression of these proteins. Immunohistochemical analysis was performed on 21 ameloblastomas, 15 keratocystic odontogenic tumors and 15 dentigerous cysts. We carried out quantitative assessment of p73 and TRAIL expression by determining the percentages of positive cells on a continuous scale. Five cases of orthokeratinized odontogenic cyst were also examined. The percentages of cells immunohistochemically positive for p73 were 52.6 ± 25.4% in ameloblastomas, 76.0 ± 13.1% in keratocystic odontogenic tumors, and 26.7 ± 30.7% in odontogenic cysts, whereas the corresponding figures for TRAIL were 57.6 ± 16.1%, 8.9 ± 10.0%, and 1.5 ± 0.5%, respectively. Imbalance of the apoptosis pathway, with dysregulation of p73 and TRAIL, seems to play a role in the oncogenesis of odontogenic tumors.

Keywords: ameloblastoma; dentigerous cyst; immunohistochemistry; keratocystic odontogenic tumor; odontogenic cyst; odontogenic keratocyst.

Introduction

Odontogenic tumors comprise a complex group of lesions arising from odontogenic tissues, with variable histopathological and clinical features (1). They range from hamartomatous and non-malignant tumors to malignant ones with metastatic capacity (2). Ameloblastoma (AM) is one of the most common odontogenic tumors, derived from the odontogenic epithelium. Its main features are slow growth and benign but locally aggressive behavior, with a high risk of recurrence (3).

Another frequent lesion is the keratocystic odontogenic tumor (KCOT), which is the second most common type of odontogenic tumor. Previously, KCOT was considered as an odontogenic cyst (OC), but in 2005 the WHO reclassified it as a benign odontogenic tumor, based on features such as its aggressive phenotypic nature, while its orthokeratinized variant has become an independent disease entity referred to as orthokeratinized odontogenic cyst (OOC) (4).

Due to the peculiarity of KCOT to form cystic lesions, differential diagnosis of this tumor from OC, in particular dentigerous cyst, can be difficult. In fact, dentigerous cyst and KCOT can share clinical and radiological features; furthermore, KCOT may have the gross appearance of a dentigerous cyst in some cases, so that a correct diagnosis can only be made by histopathological analysis (5).

Although the mechanism of oncogenesis and tumor progression remains unknown, some proteins, such as those involved in the apoptosis pathway, seem to
be involved in the differentiation and proliferation of odontogenic epithelial cells. Furthermore, apoptosis and cell proliferation seem to play a role in the pathogenesis of dentigerous cyst, maintaining the thickness of the epithelial lining (6).

Perturbation of apoptosis is evident in cancer cells, where a variety of genetic and epigenetic damage prevents this pathway from operating normally (7,8). Several studies have demonstrated alteration of the apoptosis pathway even in odontogenic tumors (9-11).

p73 belongs to the p53 family of transcription factors, which also includes p53 and p63, and shows a high degree of sequence homology (12). There are two isoforms of this protein: the transcriptional domain-containing (TAp73) and the N-terminally deleted (ΔNp73) isoforms. The first isoform induces apoptosis, while the second has anti-apoptotic activity, inhibiting the functions of both TAp73 and p53, whose expression is induced by DNA damage (12).

Increased levels of the antiapoptotic isoform of p73 have been detected in several tumors (13,14), and also recently in AM (3); Kumamoto et al. have demonstrated higher expression of ΔNp73 mRNA, suggesting that this isoform might play an oncogenic role in the odontogenic epithelium (3). Cleavage of p73 is an early event in the pathway of death receptor-mediated apoptosis, and Sayan et al. have suggested that p73 has transcription-independent functions that enhance the activity of TNF-related apoptosis-inducing ligand (TRAIL) (15).

TRAIL is a TNF-family cytokine found in a variety of tissues (16), and shows conditional expression in several immune effector cells (17-20). TRAIL can interact with DR4 and DR5, which are two functional death domain-containing membrane receptors, and also with DcR1 and DcR2, which are two decoy receptors that compete for TRAIL binding with DR4 and DR5 (21). The final result of TRAIL signaling is induction of programmed cell death though activation of the intrinsic apoptosis pathway, recruiting the inactive form of caspase-8 (22). Expression of TRAIL and its receptors has been found in several neoplasms, suggesting that the TRAIL signaling pathway is involved in endogenous tumor surveillance (22).

The expression of TRAIL in AM has been analyzed only recently, but the data regarding its distribution and correlation with the localization of apoptotic cells are discordant, and no clear correlation has emerged (22-26).

However, the overall data obtained so far suggest that TRAIL and p73 are involved in the mechanisms underlying the differentiation and proliferation of odontogenic epithelial cells, and that cell death via apoptosis has a fundamental role in oncogenesis of the odontogenic epithelium (22).

The purpose of the present study was to analyze the immunohistochemical expression of p73 and TRAIL in odontogenic tumors and cysts, and clarify how the expression of these two proteins changes in these lesions.

Materials and Methods

Tissue collection

Specimens from 51 patients (33 males and 18 females; age range 31-83 years) were retrieved from the archives of the Institute of Pathology, Marche Polytechnic University, Ancona, Italy. These included 21 solid/multicystic AMs (with a follicular growth pattern, stellate reticulum-like cell type), 15 KCOTs and 15 OCs (dentigerous cysts). None of the patients had been treated previously. All patients underwent surgical treatment with curative intent. In all cases, clinical, radiological and histological data were used to reach a final diagnosis, according to the latest WHO classification of odontogenic cysts and tumors (4). No case of KCOT was associated with genetic syndromes, precisely nevoid basal cell carcinoma syndrome (Gorlin syndrome) or Marfan syndrome. Five cases of OOC were also examined (2 males, 3 females, age range 26-65 years). This project received approval from the Ethics Institutional Board of Dental University Clinic (December 2, 2013).

Immunohistochemistry

Blocks of representative areas of cysts and tumors in each case were fixed with formalin and embedded in paraffin. Four-micrometer-thick serial sections were cut, and only those containing sufficient epithelium (1,000 cells) to evaluate antibody reactivity were considered. Immunohistochemistry was then performed on the remaining sections mounted on poly-L-lysine-coated glass slides. Deparaffinized and rehydrated sections were incubated for 30 min in 3% H2O2/methanol to quench endogenous peroxidase activity, and then rinsed for 20 min with phosphate-buffered saline (PBS) (Bio-Optica M107, Milan, Italy). Non-specific protein binding was attenuated by incubation for 30 min with 5% horse serum in PBS. Specimens were incubated overnight at 4°C with rabbit monoclonal primary anti-human p73 antibody (diluted 1:50, Abcam plc, Cambridge, UK) and mouse monoclonal anti-human TRAIL antibody (diluted 1:50, Dako, Glostrup, Denmark). The antibody was applied directly to the section and the slides were incubated overnight at 4°C in a humidified chamber. The sections were then washed three times in PBS at room temperature. Subsequently, the secondary biotinylated
Antibody was applied and detected using streptavidin peroxidase, both being incubated for 30 min at room temperature (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Following three washes with PBS, the immunoreactivity of the sections was visualized by development for two min with 0.1% 3′3-diaminobenzidine and 0.02% hydrogen peroxide (DAB substrate kit, Vector Laboratories). The sections were counterstained with Mayer’s hematoxylin solution, and then mounted in permanent mounting medium. Finally, light microscopy examination was performed. Positive control samples for p73 and TRAIL were human urinary bladder carcinoma and human normal mammary gland epithelium, respectively, which are known to show antigenic reactivity. A negative control was carried out in all cases by substituting the primary antibody with mouse serum, resulting in negative immunoreactivity for both p73 and TRAIL.

To evaluate the expression of p73 and TRAIL, the mean percentage of positive cells was determined from analysis of 300 cells in 10 random areas at high magnification. Two of the authors (C.R. and A.Z.) independently assessed the positivity for p73 and TRAIL. Quantitative assessment of p73 and TRAIL expression was performed by expressing the percentage of positive cells on a continuous scale.

Statistical analysis
Lesions were divided into three groups: AM, KCOT, and DC. GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Kruskal-Wallis test was used to evaluate the differences between groups, and statistical significance was placed at \( P < 0.05 \). Data were presented as mean ± standard deviation.

Results
p73 expression in nuclei
Immunohistochemical analysis demonstrated p73 reactivity in the nuclei of cystic and neoplastic odontogenic epithelial cells (Fig. 1A). In AM, basal cells exhibited p73 reactivity in almost all cases (Fig. 2A), and higher expression in the basal cells of KCOT (×20) (B), whereas the levels were significantly lower in OC epithelium (×20) (C).
cells was 52.6 ± 25.4%. p73 expression was significantly higher in basal cells than in central epithelium (P = 0.0001) (Fig. 1C), in agreement with other studies (3).

p73 expression was significantly higher in KCOT than in AM (P < 0.05) (Fig. 2B); quantitatively, the mean expression value for p73 was 76.0 ± 13.1%. In OC, p73 expression was significantly lower than in odontogenic tumor (P < 0.05) (Fig. 2C), with a mean value of 26.7 ± 30.7%. In OOC, p73 expression was significantly lower than in KCOT (P < 0.05).

TRAIL expression in the cytoplasm
Immunohistochemical analysis demonstrated TRAIL reactivity in the cytoplasm of the cystic and neoplastic odontogenic epithelial cells (Fig. 1B). All AM cases showed TRAIL reactivity (Fig. 3A), with a mean percentage of 57.6 ± 16.1%. The differences between the values for TRAIL in KCOT compared to AM were statistically significant (P < 0.05) (Fig. 3B), with a mean value of 8.9 ± 10.0% in the former. In OC, the levels of this protein were significantly lower (Fig. 3C) than in odontogenic tumor (mean value 1.5 ± 0.5%), and some of the samples examined showed no expression.

TRAIL expression was higher in OOC than in KCOT, but not to a statistically significant degree.

Discussion
The oncogenic processes occurring in odontogenic tumors are still unknown, but several proteins appear to be involved in the differentiation and proliferation of odontogenic epithelial cells. These proteins include some that are involved in the apoptosis pathway, such as p73 and TRAIL.

p73, along with p53 and p63, belongs to the p53 family of proteins (27), which show a high degree of sequence homology (12). Like the other p53 family members, p73 is expressed in many isomeric forms. The TP73 gene contains two alternative promoters, which generate the TAp73 and the ΔNp73 isoforms (28,29). The transactivating TAp73 promotes cell cycle arrest and programmed cell death, mimicking the activities of p53 (30), whereas ΔNp73 shows evident anti-apoptotic activity, either by forming a heteroduplex with TAp73, or by competing for DNA binding sites with both p53 and TAp73 (31-34). Genetic analyses have shown that p73 is rarely mutated or deleted (35), and recent data suggest that the role of p73 in tumor progression could be related to balance and interplay of the TAp73 and ΔNp73 isoforms (36).

In the present study, immunohistochemical evaluation of the expression of p73 in 15 cases of AM demonstrated higher expression in the basal cells, in accordance with previous data (3). However, another study had found no significant difference in TRAIL expression between the central and the basal cells (25).

Our results revealed that the expression of p73 in KCOT was significantly higher than that in AM. KCOT has a high growth potential, similar to that of AM (37). Since cancer cells show dysregulation of p73, with increased synthesis of the ΔNp73 isoform (38), this may explain the high levels found in this tumor. On the other hand, the levels of p73 in odontogenic cysts were lower than those found in AM and KCOT, in line with the concept that increased levels of p73, due to the loss of its regulation, can be regarded as an index of tumoral transformation, and therefore its levels are lower in non-tumor lesions.

Death receptor activation during programmed cell death causes cleavage of p73. The various functions of p73 include transcription-independent actions during death receptor-mediated apoptosis, which enhance the activity of TRAIL, thus suggesting that this protein also plays a role in tumorigenesis.

Although there is no clear correlation between TRAIL and odontogenic tumors, some studies have indicated that it could play an important role in the development of these lesions (25). Our results demonstrated higher expression of TRAIL in AM than in KCOT and OC, suggesting the
important role of TRAIL and its downstream pathways in neoplastic transformation, leading to the formation of AM. The low levels of TRAIL in KCOT and OC may imply that this protein and its downstream pathways may not be significantly involved in the genesis and development of these lesions.

We found that p73 showed higher expression in odontogenic tumors than in cysts, confirming the results of Kumamoto et al. (3). Furthermore, a comparison of p73 and TRAIL expression between OOC and the KCOT was performed. Although OOC and KCOT are morphologically similar, the biological behavior of these lesions differs, and OOC demonstrating less aggressive clinical behavior.

Our results revealed a significant difference in the expression of p73, confirming the literature data, and highlighting the difference between these two lesions in terms of clinical behavior, immunohistochemical profile, and surgical management.

As some previous studies have shown that p73 is cleaved by caspase-3 and caspase-8, in turn activating the TRAIL-dependent pathway (15,39), we compared the levels of expression of p73 and TRAIL proteins. The high levels of TRAIL found in AM may explain the increased expression of p73, whereas the low levels of TRAIL in KCOT suggest that the expression of p73 is not affected by TRAIL-dependent pathways, and that mechanisms other than those occurring in ameloblastic cells are involved. As expected, the low levels of p73 and TRAIL in OC suggested only minor involvement of the apoptotic pathways in these lesions, in comparison with odontogenic tumors.

In conclusion, imbalance of the apoptosis pathway, with dysregulation of some proteins such as p73 and TRAIL, seems to play a role in the oncogenesis of odontogenic tumors. However, further studies of larger series will be needed to confirm the present findings.

Conflicts of interest
None declared.

References
15. Zizzi A et al. (2013) Trail overexpression inversely correlates with histological differentiation in intestinal-type sinonasal