Immunohistochemical expression of MMPs 1, 7, and 26 in syndrome and nonsyndrome odontogenic keratocysts

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Objective. The objective of this study was to analyze the expression of matrix metalloproteinases (MMPs) 1, 7, and 26 in odontogenic keratocysts (OKCs) associated with Gorlin syndrome (SOKCs) and nonsyndrome OKCs (NSOKCs).
Study design. Twenty-one SOKCs and 20 NSOKCs were evaluated for epithelial expression of MMP-1, MMP-7, and MMP-26 and for mesenchymal expression of MMP-1 by immunohistochemistry.
Results. Strong epithelial positivity to MMP-1 was observed in 76% of SOKCs and in 15% of NSOKCs ($P < .05$). Strong mesenchymal immunoreactivity to MMP-1 was observed in 38% of SOKCs and in 20% of NSOKCs ($P > .05$). Epithelial immunoreactivity to MMP-7 was strongly positive in 67% of SOKCs and in 40% of NSOKCs ($P > .05$). For MMP-26, strong positivity was found in 62% of SOKCs, in contrast to 35% of NSOKCs ($P > .05$).
Conclusion. MMPs-1, −7 and −26 may play important roles in the biology of OKCs. Furthermore, the presence of these proteases at higher levels in SOKCs may help to explain increased OKC aggressiveness associated with nevoid basal cell carcinoma syndrome. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;106:99-105)**

The odontogenic keratocyst (OKC) has long been considered a distinct type of developmental odontogenic cyst whose probable origin is related to epithelial remnants of the dental lamina. It is distinguished from other cystic lesions by its aggressive biological behavior and recurrence tendency.[1-3]

Recent reports have shown that odontogenic keratocysts (OKCs) display high proliferative activity and protein expression associated with apoptosis inhibition,[1,3] as well as haploinsufficiency[4] and loss of heterozygosity of the tumoral suppressor gene *PTCH*.[1,5,6] These findings led the World Health Organization (WHO) to classify this cyst as a true neoplasm, giving rise to the term keratocystic odontogenic tumor.[7]

The OKC can occur sporadically or in association with the nevoid basal cell carcinoma syndrome or Gorlin syndrome, where patients present with many abnormalities, especially basal cell carcinomas, multiple OKCs, skeletal changes and dyskeratosis of the palms and soles.[5,6,8]

Analysis of the histopathology[8] and results obtained from studies on cell cycle and apoptosis,[9-11] oncogenes, tumoral suppressor genes,[11] and extracellular matrix composition[12] support the existence of a distinct biological behavior between OKCs associated with nevoid basal cell carcinoma syndrome (SOKCs) and nonsyndrome OKCs (NSOKCs). In general, SOKCs have higher growth and infiltration capacity[10] and a tendency to develop more recurrences[8] than NSOKCs do.

The interaction between cells and the extracellular matrix is critical for physiological and pathological processes.[13,14] Matrix metalloproteinases (MMPs) are enzymes that play an important role in regulating the integrity and composition of the extracellular matrix. Thus, MMPs not only promote extracellular matrix degradation but also regulate signals emitted by matrix molecules, which regulate proliferation, differentiation, and cell death.[13-15]

MMP-1 (collagenase-1, interstitial collagenase), synthesized by a wide variety of normal cells, such as fibroblasts, macrophages, and endothelial and epithelial cells, is one of the major proteases that can degrade the triple-helical domain of type I fibrillar collagen.[16-18] Type I collagen, responsible for connective tissue strength and rigidity, is the main component of the organic bone matrix.[19] A possible role for MMP-1 in keratinocyte migration[18] has been reported and the overexpression of this protease can be observed in

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Table I. Specificity, dilution, antigen retrieval, and incubation of the antibody clones

Ab clone	*Specificity*	*Dilution*	*Antigen retrieval*	*Incubation*
41-1E5*	MMP-1	1:100	Citrate pH6.0, Steamer, 30 min	Overnight (18 hours)
Ab-1/ ID2†	MMP-7	1:250	Pepsin pH1.8, oven 37°C, 60 min	Overnight (18 hours)
AHP756‡	MMP-26	1:250	Pepsin pH1.8, oven 37°C, 60 min	Overnight (18 hours)

*Calbiochem, La Jolla, CA.
†Labvision/ Neomarkers, Fremont, CA.
‡Serotec, Kidlington, Oxford, UK.

advanced epithelial malignant tumors.[20] Furthermore, MMP-1 has been implicated in tumor progression[16] and local invasiveness[21] of ameloblastomas.

Matrilysins, also called MMP-7 (matrilysin 1) and MMP-26 (matrilysin 2) proteases implicated in the substrate degradation of basement membranes, are involved in a variety of processes, such as cell proliferation,[22] apoptosis,[23] invasion, and metastasis.[14,24-26] Matrilysins differ from other MMPs in their low molecular weight, by their lack of a C-terminal hemopexin domain common to other MMPs,[26,27] and their conspicuous expression in epithelial cells.[28,29] Despite reports describing the importance of matrilysins in normal glandular epithelium[22,25,29] as well as in malignant epithelial neoplasms,[24,28,29] to date, there are no studies on the expression of MMPs-7 and −26 in odontogenic lesions.

In light of the importance of MMPs in regulating the integrity and composition of the extracellular matrix and in view of reports suggesting a distinct biological behavior between SOKCs and NSOKCs, the purpose of this study was to analyze the immunohistochemical expression of MMPs-1, −7, and −26 in OKCs specimens associated or not to the nevoid basal cell carcinoma syndrome. We hope to contribute to understanding the differences in the biological behavior of SOKCs and NSOKCs described in the literature.

MATERIALS AND METHODS

Forty-one specimens of OKCs, 21 of SOKCs, and 20 of NSOKCs, obtained from the Oral Pathology Departments of the Federal University of Rio Grande do Norte (UFRN) and of the University of Fortaleza (UNIFOR), were randomly selected for this study. The sample size for this study was limited to available institutional archival cases. The cases of SOKC and NSOKC were not matched for age, sex, or anatomic location. In all cases, the histological diagnosis was based on the second WHO classification.[30] All the syndrome patients had been diagnosed according to the criteria proposed by Kimonis et al.[31] and presented with multiple OKCs. The patients with sporadic OKCs had single lesions and had been clinically and radiographically assessed to exclude the presence of other Gorlin syndrome manifestations. Moreover, in both groups the cysts were de novo lesions. Serial sections, 3-μm thick, were taken from the tissue blocks and processed for immunohistochemical examination. The study was approved by the Research Ethics Committee of the University of Fortaleza, Fortaleza, Brazil.

Immunohistochemical methods

The tissue sections were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were then washed in phosphate-buffered saline (PBS). The antigen retrieval, antibody dilution and clone type for MMPs-1, −7, and −26 are shown in Table I. After treatment with normal serum, the sections were incubated in a moist chamber with primary antibodies. The sections were then washed twice in PBS and treated with streptoavidin-biotin-peroxidase complex method (Dako, Glostrup, Denmark) at room temperature in order to bind the primary antibodies. Peroxidase activity was visualized by immersing tissue sections in diaminobenzidine (D5637; Sigma Chemical, St. Louis, MO), resulting in a brown reaction product. Finally, tissue sections were counterstained with Mayer's hematoxylin and coverslipped. Positive controls for MMPs-1, −7, and −26 were sections of normal duodenum, ovarian cancer, and endometrial carcinoma, respectively. As negative controls, samples were treated as above, except that the primary antibody was replaced by a solution of bovine serum albumin (BSA) in PBS.

Immunostaining assessment and statistical analysis

After immunohistochemical treatment, the tissue sections were examined by light microscope using ×10 and ×40 objectives. Two oral pathologists, in a double-blind study, verified the presence or absence of immunoreactivity to MMPs-1, −7, and −26 in the cytoplasm of epithelial cells, characterizing it, according to Kumamoto et al.,[16] as (−) negative, (+) positive, and (++) strongly positive. Additionally, owing to the conspicuous expression of matrilysins in epithelial cells,[28,29] the immunoreactivity in the cytoplasm of mesenchymal

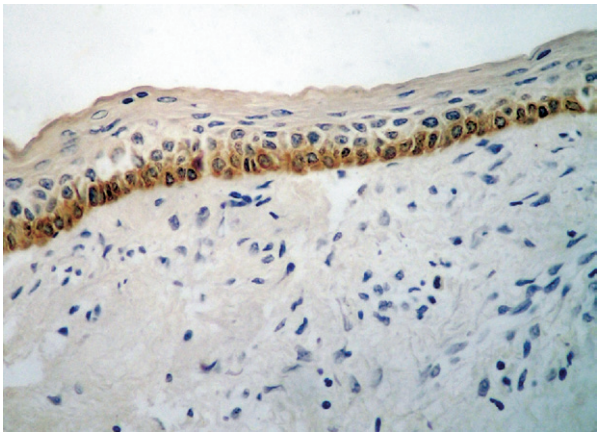

Fig. 1. SOKC exhibiting strong epithelial positivity (++) to MMP-1 mainly located in the basal cell layer (SABC method, original magnification ×400).

cells of the connective tissue was studied only for MMP-1, using the same scores applied to the analysis of epithelial cells.

The results obtained were submitted to statistical analysis. Computations were made using the Statistical Package for the Social Sciences (version 10.0; SPSS Inc., Chicago, IL). Although descriptive analysis was performed in all SOKCs and NSOKCs, only the immunoreactive cases were selected for statistical analysis. To analyze the immunoreactivity of epithelial and mesenchymal cells to MMPs-1, −7, and −26, nonparametric Fisher's exact test was performed at a significance level of .05 ($P < .05$).

RESULTS

The assessment of the epithelial immunoreactivity to MMP-1 showed that 100% of the SOKCs were immunoreactive, most of them (76%) with strong positivity (Fig. 1). Among the NSOKCs, 90% of the cases had epithelial immunostaining, with only 15% of the sample showing strong positivity. The difference between SOKC and NSOKC cases, indicating epithelial immunoreactivity to MMP-1, was statistically significant ($P < .05$) (Table II). Cytoplasm immunoreactivity of mesenchymal cells was observed in 100% of SOKCs (Fig. 2), with 38% of the cases strongly positive. Additionally, immunoreactivity in the mesenchymal cells was found in 60% of NSOKCs, with strong positivity observed in 20% of the cases. Fisher's exact test showed no statistically significant difference between the groups ($P > .05$) (Table II).

The analysis of the epithelial expression of MMP-7 showed that 100% of SOKCs were immunoreactive to this protease, with strong positivity in 67% of the sample. With respect to NSOKCs, 75% of the sample showed epithelial immunoreactivity to MMP-7 (Fig. 3), with only 40% of the cases strongly positive. The statistical analysis between SOKC and NSOKC cases showing epithelial immunoreactivity to MMP-7 revealed no significant difference ($P > .05$) (Table II). Despite the conspicuous epithelial immunoexpression of MMP-7 in OKCs, few mesenchymal cells from the connective tissue of SOKCs and NSOKCs showed mild cytoplasmatic immunoreactivity to this protease. Nevertheless, this scant immunoreactivity was not sufficient to be considered as a positive pattern, according to the parameters used in our research.

In regard to the epithelial immunoreactivity to MMP-26, 100% of SOKCs were positive, with a strong pattern present in 62% of the sample (Fig. 4). In NSOKCs, 80% of the specimens had epithelial immunoreactivity to MMP-26, with strong positivity in 35% of the cases. Statistical analysis of SOKC and NSOKC cases with epithelial immunoreactivity to MMP-26 showed no statistically significant difference ($P > .05$) (Table II). As in the MMP-7 analysis, mild cytoplasmatic immunoreactivity to MMP-26 was observed in a few of the mesenchymal cells present in SOKC and NSOKC connective tissue. This scant immunoreactivity was not sufficient to be considered as a positive pattern, according to the parameters used in our research.

DISCUSSION

It has been reported that OKCs associated with nevoid basal cell carcinoma syndrome have a distinct biological behavior when compared to sporadic OKCs.[1,12] Despite these reports, few studies have been conducted to confirm this observation. Descriptions of the histopathology[8] and results obtained from studies on the cell cycle and apoptosis,[9-11] oncogenes, tumoral suppressor genes,[11] and extracellular matrix components[12] have also identified differences between aggressive SOKCs and less-aggressive NSOKCs.

However, few studies have evaluated the presence of MMPs in OKCs. Many of these have been concerned with identifying MMPs-1, −2, −8, and −13[32-34] in OKCs alone or with comparing them to other odontogenic cysts, such as radicular and dentigerous cysts. To date, this is the first study to assess comparatively the immunoreactivity to MMPs-1, −7, and −26 between SOKCs and NSOKCs.

OKCs are primarily intraosseous lesions, where the surrounding osteoid extracellular bone matrices are destructed.[30] Type I collagen, responsible for connective tissue strength and rigidity, is the main bone organic matrix component[19] and MMP-1 is one of the proteases that can degrade the triple-helical domain of type I fibrillar collagen.[16-18]

Table II. Immunoreactivity to matrix metalloproteinases in odontogenic keratocysts

	No. of cases	MMP-1			P	MMP-7			P	MMP-26			P
		(−)	(+)	(++)		(−)	(+)	(++)		(−)	(+)	(++)	
Epithelial cells													
SOKCs	21	0 (0)	5 (24)	16 (76)	.001	0 (0)	7 (33)	14 (67)	.499	0 (0)	8 (38)	13 (62)	.331
NSOKCs	20	2 (10)	15 (75)	3 (15)		5 (25)	7 (35)	8 (40)		4 (20)	9 (45)	7 (35)	
Fibroblasts													
SOKCs	21	0 (0)	13 (62)	8 (38)	1.000								
NSOKCs	20	8 (40)	8 (40)	4 (20)									

Immunohistochemical reactivity: (−) negative, (+) positive, (++) strongly positive.
Values in parentheses are in percent.
Statistical significance: $P < .05$.
MMP, matrix metalloproteinase; SOKCs, odontogenic keratocysts associated with nevoid basal cell carcinoma syndrome; NSOKCs, nonsyndrome odontogenic keratocysts.

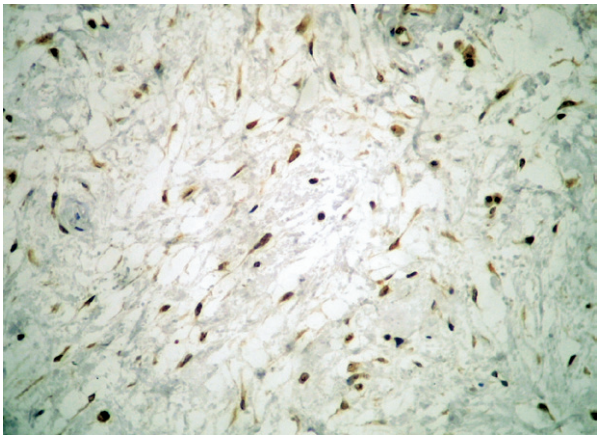

Fig. 2. Mesenchymal cells of SOKC revealing cytoplasmatic positivity (+) to MMP-1 (SABC method, original magnification ×400).

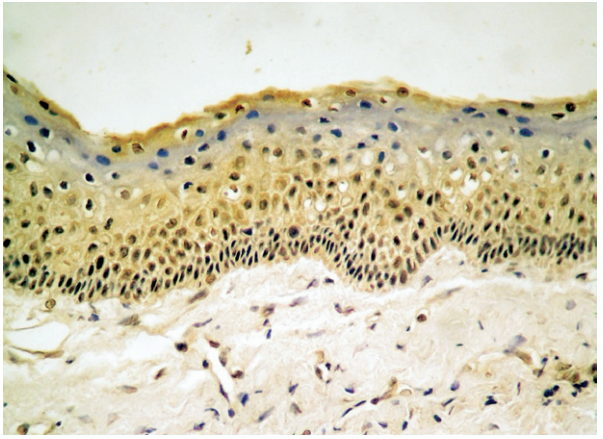

Fig. 3. Positive immunostaining (+) to MMP-7 located in all epithelial layers of NSOKC (SABC method, original magnification ×400).

In our study, the high proportion of SOKCs and NSOKCs with both epithelial and mesenchymal cell immunoreactivity to MMP-1 strongly suggests a role of this protease in the OKC biology. The presence of MMP-1 in OKCs may be associated with the degradation of the organic bone matrix,[19] favoring OKC dissemination through the trabecular spaces. Moreover, the highest epithelial immunoreactivity to MMP-1 in SOKCs ($P < .05$) may help to explain the increased aggressiveness of this lesion, when associated with nevoid basal cell carcinoma syndrome, reported in the literature.[8,10,12] The synthesis and secretion of the proenzyme form of MMP-1 by cultured fibroblasts from OKCs[34] and reports implying a role for MMP-1 in tumor progression[16] and local invasiveness of ameloblastomas,[21] as well as in keratinocyte migration,[18] invasion, and metastasis[20] reinforce the importance of this protease in the OKC biology.

While our results regarding epithelial immunoexpression of MMP-1 may help to explain the increased aggressiveness of SOKCs, it is too early to suggest that if a patient's OKC demonstrates strong epithelial immunoreactivity to MMP-1, the patient should be notified that he or she most likely has the nevoid basal cell carcinoma syndrome. Therefore, additional studies are needed to clarify a possible diagnostic utility for MMP-1 epithelial immunoreactivity in OKCs.

Pinheiro et al.,[21] using immunohistochemical, zymographic, and western blot analysis, showed that parenchymal and stromal cells of ameloblastoma can produce MMPs-1, −2, and −9. In addition, these authors found increased proliferation indexes of ameloblastoma cells in close proximity to bone, assessed by the Ag-NOR technique, suggesting an interdependent mechanism involving the action of MMPs and the proliferative activity of ameloblastomas. Despite the identification of immunoreactivity to MMP-1 only in stromal cells of ameloblastomas, Kumamoto et al.[16]

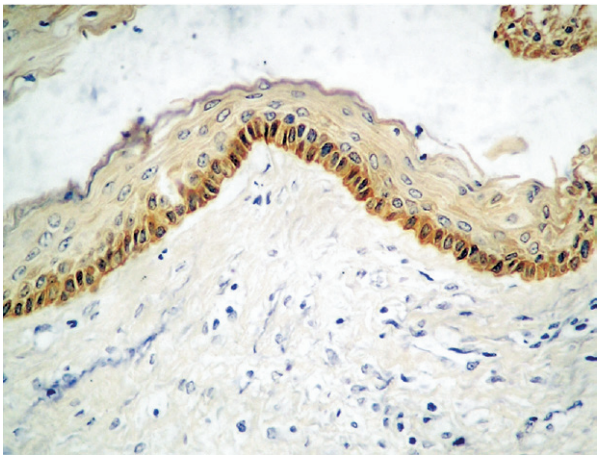

Fig. 4. Strong epithelial positivity (++) to MMP-26 in SOKC observed predominantly in the basal cell layer (SABC method, original magnification ×400).

suggest that MMP-1 production by these cells might be associated with tumor cell progression of ameloblastomas.

Based on the findings reported by Kumamoto et al.[16] and Pinheiro et al.,[21] it could be suggested that the MMP-1 expression by OKCs cells observed in our study might be related to a functional role for this protease beyond simple extracellular matrix degradation. In fact, MMP-1 activity is capable of releasing growth factors and cytokines present in the surrounding organic bone matrix.[19,21] The released factors could contribute to the higher epithelial proliferative indexes observed in OKCs when compared to other odontogenic cysts.[1,3,11]

Remodeling the epithelial basement membrane is a crucial step in many processes, from injury healing and keratinocyte migration[20] to tumor invasion and metastasis.[15] Many extracellular matrix substrates for matrilysins are epithelial and vascular basement membrane components, such as, laminin, fibronectin, and type IV collagen.[2,12,35] Thus, the immunoreactivity of the SOKC and NSOKC epithelial cells to MMPs-7 and −26 observed in this research corroborate the results reported by other studies that analyzed basement membrane composition in the OKC epithelial-connective tissue junction.

Oliveira et al.[2] and Poomsawat et al.[35] verified a weak and discontinuous immunoreactivity to type IV collagen in the OKC epithelial basement membrane, when compared to other odontogenic cysts, such as radicular and dentigerous cysts. This finding led the authors to suggest that type IV collagen discontinuity, together with altered expression of other extracellular molecules in the OKC epithelial basement membrane could be involved in the aggressive biological behavior of this lesion. The presence of matrilysins in the SOKC and NSOKC epithelial component observed in our study may account for the weak and discontinuous pattern of type IV collagen expression along OKC epithelial basement membranes, as reported by Oliveira et al.[2] and Poomsawat et al.[35]

Despite the absence of statistical differences ($P > .05$) in epithelial immunoreactivity to MMPs-7 and −26 between SOKCs and NSOKCs, our results showed a tendency for a more consistent expression of these proteases in SOKCs. Comparing the samples, 100% of the SOKC cases showed epithelial immunoreactivity to MMPs-7 and −26, whereas only 75% and 80% of NSOKC cases had epithelial immunohistochemical expression of matrilysins 1 and 2, respectively. Furthermore, when compared to NSOKCs, the SOKCs had a higher proportion of cases with strong epithelial positivity for MMPs-7 (67% versus 40%) and −26 (62% versus 35%).

Our results in relation to epithelial immunoreactivity to MMPs-7 and −26 in SOKCs and NSOKCs agree with those of Amorim et al.,[12] who found a weaker immunohistochemical expression of type IV collagen along the SOKC basement membrane when compared to NSOKCs. Therefore, the slightly higher percentage of SOKCs showing strong epithelial immunoreactivity to matrilysins corroborates literature reports that suggest increased aggressiveness of the OKCs associated with nevoid basal cell carcinoma syndrome.[8,10,12]

Nevertheless, other studies need to be conducted to confirm if the differences in the expression of MMPs-1, −7, and −26 between SOKCs and NSOKCs could be related to more aggressive biological behavior. Thus, clinical and radiographical follow-up data are fundamental. Unfortunately, this information was not available to our study.

Besides degrading basement membrane components, MMPs-7 and −26 are capable of activating other MMPs, such as MMP-2 (only MMP-7) and MMP-9 (both MMPs-7 and −26).[26,27] MMPs-2 and −9, also known as gelatinases, are important proteases that degrade the components present both in basement membranes, such as type IV collagen and laminin,[15,36] and in the interstitial extracellular matrix, particularly the fragments derived from the proteolysis of type I fibrillar collagen, denominated gelatins.[18,36]

MMP-2 has been observed by immunohistochemistry in OKC epithelial basement membranes,[33] by in situ hybridization in OKC connective tissue fibroblasts,[33] by western immunoblotting and gelatin zymography in cultured fibroblasts,[32] and in co-cultured OKC epithelial cells and fibroblasts.[34] In light of these findings, MMP-2 has been implied in the degradation of the

extracellular matrix surrounding OKCs. It also favors epithelial migration and OKC growth.[33]

Based on the reports revealing an important role for MMP-2 in OKC biology,[33,34] the immunohistochemical expression of MMP-7 in SOKCs and NSOKCs observed in our study shows a possible additional importance of this protease. The presence of matrilysin 1 in OKCs may contribute to the activation of proenzyme forms of MMP-2,[26,27] which could degrade the surrounding extracellular matrix and favor epithelial migration and OKC growth.[33,34]

The possibility of interactions between WNT/β-catenin and PTCH/SHH intracellular signaling pathways might be one of the causes of the higher immunohistochemical expression of MMPs-7 and −26 in SOKCs. Recent studies in neoplasms associated with the Gorlin syndrome, such as medulloblastomas[37] and craniopharyngiomas,[38] showed that *PTCH* gene mutations were associated with an overexpression of several components of the WNT intracellular signaling pathway, mainly β-catenin.

Accordingly, β-catenin is able to bind to the T-cell factor (TCF) family of transcription factors and subsequently activate target genes, including MMPs-7 and −26 genes. Therefore, deregulation of the WNT signaling pathway is implicated in the overexpression of matrilysins in many epithelial neoplasms, such as colorectal and esophageal carcinomas, contributing to local invasiveness and metastasis.[24,25,29]

Despite the conspicuous expression of MMPs-7 and −26 in epithelial neoplasms,[28,29] our results showed a mild immunoreactivity to these proteases in the cytoplasm of few mesenchymal cells in SOKC and NSOKC connective tissues. Unfortunately, this scant immunoreactivity was not sufficient to be considered as a positive pattern, according to the parameters used in our research. Nevertheless, this observation might suggest a possible role for matrilysins in degrading other extracellular matrix substrates, such as tenascin and gelatins, as reported by Chakraborti et al.[13] and Nabeshima et al.[15] Therefore, additional studies are needed to clarify the possible implication of matrilysins in biological processes that differ from their main action on basement membranes, such as the degrading and remodeling of the interstitial matrix.

Taken together, our results suggest an important role for MMPs-1, −7, and −26 in OKC biology. Furthermore, the presence of these proteases at higher levels in SOKCs may help to explain increased OKC aggressiveness associated with nevoid basal cell carcinoma syndrome.

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