Transformation-specific matrix metalloproteinases, MMP-7 and MMP-13, are present in epithelial cells of keratoacanthomas

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Keratoacanthomas are rapidly growing hyperproliferative skin tumors that may clinically or histologically be difficult to distinguish from well-differentiated squamous cell cancers (SCCs). UV light, trauma, and immune suppression represent their etiological factors. As matrix metalloproteinases (MMPs) are implicated at all stages of tumorigenesis, we investigated the expression profile of several cancer-related MMPs to find markers that would differentiate keratoacanthomas from SCCs and shed light to the pathobiology of keratoacanthoma. Samples from 31 keratoacanthomas and 15 grade I SCCs were studied using immunohistochemistry for MMP-2, -7, -8, -9, -10, -13, and -19 and p16 and laminin-5 γ 2 chain. *In situ* hybridization for MMP-7, -10, and -13 was performed in a subset of tumors. Keratinocytes with atypia, presence of neovascularization, and composition of the inflammatory infiltrate were graded from hematoxylin–eosin stainings. MMP-7 was present in the epithelium of 4/31 keratoacanthomas and 9/15 SCCs, MMP-8 in 3/30 keratoacanthomas and 0/15 SCCs, but MMP-13 in 16/31 keratoacanthomas and 10/15 SCCs, and MMP-10 in 28/31 keratoacanthomas and all cancers. MMP-9 was detected in the epithelium in 5/31 keratoacanthomas and 8/15 SCCs, whereas MMP-2 was only present in fibroblasts in both tumors. MMP-19 was upregulated in proliferating epithelium of keratoacanthomas as was p16. Cytoplasmic laminin-5 γ 2 was particularly abundant in keratinocytes at the pushing border of MMP-13-positive keratoacanthomas. We conclude that although some MMPs (MMP-10 and -13) are abundantly expressed in keratoacanthomas, the presence of MMP-7 and -9 in their epithelial pushing border is rare and should raise suspicion of SCC. Further, the loss of MMP-19 and p16 could aid in making the differential diagnosis between well-differentiated SCC and keratoacanthoma. Frequent expression of the transformation-specific MMP-13 in keratoacanthomas suggests that they are not benign tumors but incomplete SCCs.

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Keratoacanthomas are squamous neoplasms most likely derived from hair follicle cells on sun-exposed skin.¹ They present clinically with a rapid growth phase for the first 4–8 weeks and a possible spontaneous self-induced regression after 3–6 months.² The origin of keratoacanthomas is uncertain. UV light may be of etiologic significance as

well as some chemical agents, immune suppression, and trauma.^{1,3}

Differential diagnosis between keratoacanthomas and well-differentiated squamous cell cancers (SCCs), based on clinical and histomorphological data, may be problematic and there are some reports on lesions classified as keratoacanthoma that have metastasized.⁴ It is thought that those keratoacanthomas of sun-exposed locations and diagnosed in older patients may more frequently progress to SCC.⁵ Some authors believe that keratoacanthoma is a well-differentiated variant of SCC representing a range of neoplastic activity with no clear distinction, whereas some authors consider the histological and cytological differentiation precise, sure, and

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possible.^{1,5-7} Thus, the natural behavior of keratoacanthomas remains unclear and controversial, and practically all these tumors are currently surgically excised.³ In addition to the proportion of cells with atypia, one malignancy-associated criterion in keratoacanthomas is increased microvessel density.⁵

Matrix metalloproteinases (MMPs), a family of 23 human zinc-dependent proteolytic enzymes, have been implicated at all stages of tumorigenesis.⁸ They mediate apoptosis, proliferation, and differentiation, tumor angiogenesis, and malignant conversion⁹ by degrading basement membranes and various matrix components as well as by activating chemokines and growth factors. Particularly MMP-1, -2, -7, -9, -13, -14, and -26 have been shown to be present in well-differentiated cutaneous SCC.¹⁰⁻¹² Paradoxically, recent data show that some novel MMPs are in fact instrumental for normal keratinocyte proliferation (MMP-19 and -28) and disappear in cancer,^{13,14} whereas MMP-3, -7, -9, and -12 effectively cleave plasminogen to angiostatin and type XVIII collagen to endostatin, leading to inhibition of angiogenesis and eventually retardation of tumor growth.^{15,16} Our recent results suggest that epithelial expression of MMP-7, -12, and -13, but not that of MMP-1, -3, -8, -9, and -10, in chronic wounds

provides a diagnostic clue for distinguishing well-differentiated SCCs from nonmalignant wounds.¹⁷

It is of major interest to find markers that distinguish keratoacanthoma from SCC and to better define the pathobiologic steps in its development. The aim of this study was to investigate whether the pattern of epithelial MMP expression can differentiate keratoacanthomas from SCCs. Furthermore, we also stained for two other cancer-related molecules, laminin-5 γ 2 and p16. Laminin-5 γ 2 serves as migrating substrate in neoplasia and stimulates invasion after having been cleaved by, for example, MMP-2,^{18,19} whereas mutational loss of p16 is a frequent early event in the development of SCC.²⁰

Materials and methods

Patients and Tissues

Formalin-fixed, paraffin-embedded samples from 31 patients with keratoacanthoma (Table 1) and 15 patients with grade I SCC (Table 2) were obtained from the Departments of Dermatopathology and Pathology, University of Helsinki, Finland. Three of our patients were immunosuppressed following organ transplantation. Diagnoses were made by an

Table 1 Epithelial expression pattern of MMPs and other proteins in KAs

Patient	Age/sex	Area	Infl.	Atypia	Angiog.	MMP-7	MMP-8	MMP-9	MMP-10	MMP-13	MMP-19	LN5	P16
1 ^a	83/M	Neck	5	2	1	—	+ ^b	+	+++	++	+	3	1
2 ^a	81/F	Arm	4	0	2	—	—	—	+	++	+	1	2
3 ^a	46/M	Nose	2	1	1	—	— ^b	—	+	++	+	2	2
4 ^a	73/M	Temple	3	2	1	+	— ^b	—	+	++	+	2	0
5 ^c	89/F	Leg	6	0	3	—	— ^b	—	—	++	+	1	1
6 ^c	55/F	Lip	6	0	3	++	— ^b	—	—	++	+	2	1
7 ^a	76/F	Cheek	4	0	2	—	— ^b	+	++	+	++	2	2
8 ^a	75/M	Cheek	6	0	1	—	— ^b	—	+	+	++	1	2
9 ^a	54/F	Arm	2	0	0	—	—	—	+	+	+	1	2
10 ^a	81/M	Cheek	4	0	1	—	+	—	++	+	+	2	0
11 ^a	57/F	Cheek	4	0	1	—	— ^b	—	+	+	+	2	1
12 ^a	50/M	Lip	3	0	1	—	— ^b	+	++	+	—	1	1
13 ^a	69/F	Leg	2	0	1	—	—	—	+	+	+	2	2
14 ^c	63/F	Nose	3	1	1	—	—	—	+	+	+	1	3
15 ^a	86/F	Forehead	2	0	1	—	— ^b	+	+	+	—	1	1
16 ^a	56/F	Eyelid	2	0	0	—	—	—	—	+	+	2	1
17 ^c	62/M	Abdomen	2	1	1	—	+	—	+	—	+	2	3
18 ^a	53/F	Arm	2	0	0	—	ND	—	++	—	+	1	2
19 ^a	74/F	Abdomen	2	0	0	—	—	—	+	—	+	1	2
20 ^c	60/F	Nose	4	1	1	—	— ^b	—	+	—	—	2	2
21 ^c	89/F	Cheek	5	1	1	—	—	—	+++	—	+	1	0
22 ^c	72/M	Lip	2	0	2	—	—	—	+	—	+	1	3
23 ^c	61/M	Chest	4	1	1	—	— ^b	+	+++	—	—	2	2
24 ^a	79/M	Back	4	0	0	—	— ^b	—	+	—	+	2	2
25 ^a	73/F	Forehead	5	0	1	+	—	—	+	—	+	1	2
26 ^a	85/F	Temple	2	0	0	ND	—	—	+	—	+	1	2
27 ^a	55/F	Arm	3	0	0	—	—	—	+	—	+	2	3
28 ^c	74/F	Temple	6	0	1	+	—	—	+	—	+	1	1
29 ^c	58/F	Temple	6	0	3	—	—	—	+	—	+	0	0
30 ^a	79/M	Forehead	3	0	1	—	— ^b	—	++	—	+	0	2
31 ^a	74/F	Cheek	3	0	0	—	—	—	+	—	+	2	1

^aClassical KA with symmetrical structure and sharply demarcated pushing border.

^bPositive neutrophils, ND = not determined.

^cUnclassical KA with asymmetrical structure and unevenly demarcated pushing border. Infl. = inflammation; Angiog. = angiogenesis; LN5 = laminin-5 γ 2; KA = keratoacanthomas; MMP = matrix metalloproteinase.

Table 2 Epithelial expression of MMPs in KAs and well-differentiated cutaneous SCCs

	MMP-2	MMP-7	MMP-8	MMP-9	MMP-10	MMP-13	MMP-19	p16
KA	0/31	4/31	3/30	5/31	28/31	16/31	27/31	27/31
SCC	0/15	9/15*	0/15	8/15*	15/15	11/15	6/15**	7/15*

* $P < 0.01$, ** $P < 0.001$.

KA = keratoacanthoma; SCC = squamous cell carcinoma; MMP = matrix metalloproteinase.

experienced dermatopathologist (LJ). The histological criteria used for keratoacanthoma were a cup-shaped architecture with central keratin plug, basaloid layer in proliferating endophytic lobules, large cells with paler eosinophilic cytoplasm, lack of anaplasia, and a sharp outline between tumor nests and the stroma.²¹ As assessed by hematoxylin–eosin staining, the keratoacanthoma samples were characterized by their histologic structure,²² variable numbers of atypical cells, neovascularization, and inflammation (Table 1). The number of atypical cells was graded as 0 (less than 25% cells with atypia of the total number epidermal cells in the tumor), 1 (25–50% of the total number of epidermal cells in the tumor showing atypia), or 2 (50–100%). Neovascularization at the pushing border was graded as 0 (density of capillaries the same as in normal dermis), 1 (0–25% more capillaries than in normal dermis), 2 (25–50%), and 3 (over 50%). Grading for inflammation was based on the number of lymphocytes, neutrophils, and macrophages as assessed by an experienced dermatopathologist and scored as 0–2 (scarce), 3–4 (moderate), or 5–6 (abundant). The use of archival specimens was approved by the corresponding ethical committee of the Helsinki University Central Hospital, Finland.

Immunohistochemistry

Immunostaining of the sections was performed by the avidin–biotin–peroxidase complex technique (Vectastain ABC Kit; Vector Laboratories, Inc, Burlingame, CA, USA). Diaminobenzidine or aminoethylcarbazole were used as chromogenic substrates and Mayer hematoxylin was used as a counterstain. Endogenous peroxidase was blocked by incubation with 3% H₂O₂ for 10 min at room temperature. If necessary, sections were pretreated with 10 mg/ml trypsin, incubated in a +95°C water bath or microwaved in citrate buffer. We used mouse monoclonal antibodies to study the localization of MMP-2 (1:100; IM33L, Oncogene Research Products, San Diego, CA, USA), MMP-7 (1:70; IM40L, Oncogene), MMP-8 (1:10; IM38L; Oncogene), MMP-9 (1:50; GE-213, Research Diagnostics, Flanders, NJ, USA), MMP-10 (1:200; 5E4, Novocastra Laboratories, Newcastle, UK), MMP-13 (1:40; IM64L, Oncogene), and p16^{INK4} (1:350; G175-405; BD Biosciences, San Jose, CA, USA).¹⁷ Rabbit polyclonal antiserum against human MMP-19 (PC374;

Oncogene)¹⁴ was diluted 1:40 and laminin-5γ2 (gift from Professor Karl Tryggvason, Karolinska Institutet, Sweden) 1:1000 in 1% BSA.¹⁸ Controls were performed with mouse immunoglobulins or with rabbit preimmune serum. Semiquantitative immunoreactivity grading for MMPs was based on positive cytoplasmic staining at the pushing border and scored as negative (0–5 positive cells), + (5–50 positive cells), ++ (50–100 positive cells), and +++ (over 100 positive cells). Stainings for p16 and laminin-5 were graded as follows: 0 (no staining), 1 (focal, weak staining), 2 (diffuse, moderate staining), and 3 (diffuse, strong staining). The slides were analyzed independently by two investigators (TK and US-K).

In Situ Hybridization

The production and the specificity of the antisense and sense human MMP-7, -10, and -13 probes have been described previously.^{23–25} *In situ* hybridization was performed on 5-μm sections as described in detail²⁶ to confirm immunohistochemical findings. Sections were hybridized with ³⁵S-labeled probes (3 × 10⁴ c.p.m./μl of hybridization buffer) at 50°C for at least 18 h in a humidified chamber. After 20–50 days of autoradiography, the photographic emulsion was developed, and the slides were stained with hematoxylin and eosin. Samples previously positive (SCCs) for the studied mRNAs were used as positive controls.¹⁷ No signal was detected with the sense probe.

Statistical Analysis

Unpaired two-tailed *t*-test was performed to compare the number of positive cells in p16 and laminin-5 samples in MMP-13-positive and MMP-13-negative groups. χ^2 test was employed to compare the number of MMP-positive samples in keratoacanthomas vs SCCs (Table 2). *P*-value under 0.05 was considered as significant.

Results

Matrilysin-1

MMP-7 protein was detected in epithelial cells of 4/31 keratoacanthomas and 9/15 SCCs (Figure 1a and b) (Tables 1 and 2). Neovascularization was

Figure 1 Immunohistochemistry for MMP-7, MMP-13, and MMP-8 in keratoacanthoma and SCC. (a) Expression of MMP-7 (arrows) by keratinocytes in keratoacanthoma. (b) MMP-7-positive keratinocytes in SCC. (c) MMP-13 expression at the pushing border in keratoacanthoma. (d) Epithelial expression of MMP-13 in SCC. (e) Expression of MMP-8 in keratinocytes in keratoacanthoma. Positive neutrophils (arrow) were detected in half of the keratoacanthoma samples. Scale bars: 125 μm (a, b), 50 μm (c, d), 25 μm (e).

observed in all MMP-7-positive keratoacanthoma samples, but the presence of MMP-7 did not associate with keratinocyte atypia or inflammation (Table 1).

Collagenases

MMP-13 was expressed by basal keratinocytes in 16/31 keratoacanthomas and 11/15 SCCs (Figure 1c and d). No correlation of MMP-13 expression with atypical cells in keratoacanthomas was noted (Table 1), but there was a trend for more abundant angiogenesis in MMP-13-positive samples (1.25 ± 0.77 vs 0.80 ± 0.96) (Table 1). Epithelial expression for MMP-8 was detected in 3/30 keratoacanthomas (Figure 1e), but only MMP-8-positive neutrophils were seen in 5/15 SCCs (Tables 1 and 2). MMP-8-positive neutrophils were detected in 14/32 keratoacanthomas usually localized in the upper parts of the tumors rather than near the pushing epithelial border (Table 1). All MMP-8-positive keratoacanthomas had neovascularization (Table 1).

Gelatinases

In 5/31 keratoacanthomas and 8/15 SCCs, MMP-9 expression was seen in a small number of epithelial cells at the pushing border (Figure 2a and b). In 22/31 keratoacanthomas, MMP-9 was produced only by macrophages and neutrophils (data not shown). Epithelial MMP-9 expression did not associate with keratinocyte atypia or angiogenesis in keratoacanthomas (Table 1). MMP-2 protein was not detected in the epithelium of any keratoacanthoma or SCC, but was abundantly expressed by fibroblasts in all the samples (Figure 2c).

MMP-19 and MMP-10

MMP-19 was generally present in epithelial keratinocytes at the pushing border of keratoacanthomas (Figure 2d) (Table 1), but only in 6/15 SCCs (Table 2). In SCCs, MMP-19 was expressed by normal hyperproliferative epidermis, but generally disappeared from the invasive cancer cell nests (Figure 2e and f). MMP-10 was expressed in the basal epithelial cells of the pushing border in 28/31 keratoacanthomas and in the basal epithelium of cancer cell nests in all SCCs (Figure 3a and b; Tables 1 and 2).

Tumor Suppressor p16

The expression of p16 tumor suppressor protein was recently shown to be induced in cells of some premalignant lesions and consistently during the early stage of SCC invasion.²⁷ In keratoacanthomas, p16 was detected in hyperproliferating areas of the tumors independent of the number of atypic

keratinocytes or the degree of inflammation (Figure 3c) (Table 1). However, some specimens had clearly fewer p16-positive cells than others (Table 1). P16 expression was stronger in MMP-13-negative samples (1.37 ± 0.81 vs 1.8 ± 0.94). In SCCs, p16 was mostly absent from the invasive cancer cell nests (Figure 3d) and only 7/15 samples had occasional p16-positive cells at the invasive front (Table 2).

Laminin-5 γ 2

The number of keratinocytes at the pushing border with cytoplasmic staining for laminin-5 γ 2 was variable (Table 1) (Figure 3e and f). There was a trend for stronger laminin-5 γ 2 expression in MMP-13-positive samples (1.63 ± 0.63 vs 1.27 ± 0.70). In most samples, laminin-5-positive keratinocytes colocalized with MMP-10-positive cells at the pushing border (Table 1), although MMP-10 was much more widely expressed (data not shown). There were no differences in the MMP, p16, and laminin-5 profiles of patients with immunosuppression compared to the others.

Discussion

During transformation into an invasive tumor, epithelial cells undergo alterations in morphology and adhesive properties, resulting in loss of normal epithelial differentiation and a switch to a more motile phenotype. Individual MMPs have unique functions during tumorigenesis and their presence in cancer tissue can be used as prognostic marker to predict tumor invasiveness.²⁸ Therefore, we aimed to investigate the expression pattern of MMPs and their possible role as diagnostic indicators in keratoacanthomas. Very little data exist on MMPs in keratoacanthomas in contrast to SCC. MMP-1 was reported to be present in tumor cells and adjacent fibroblasts in keratoacanthoma²⁹ and the authors concluded that it participates in the transepithelial migration of elastic fibers typical for this tumor. Furthermore, MMP-11 was detected in fibroblasts surrounding 22% of examined keratoacanthomas.³⁰ We have recently shown that also MMP-26 is present in the pushing border of keratoacanthomas.¹¹

As the expression of MMP-2, -7, -8, and -13 has previously been detected in malignantly transformed human keratinocytes^{10,17,31,32} in SCCs, but not in normal migrating keratinocytes, we chose them to begin with. Furthermore, in an epithelial carcinogenesis model of HPV16/MMP-9 $-/-$ mice, MMP-7 and MMP-13 are only seen in frank carcinoma³³ and in tumors both have been detected exclusively in malignantly transformed squamous cell carcinoma cells but not in premalignant lesions.³⁴ MMP-7 contributes to the initiation of epithelial-to-mesenchymal transition by cleavage of E-cadherin.^{33,35} In keratoacanthomas, MMP-7

Figure 2 Expression of MMP-2, -9, and -19 in keratoacanthoma and SCC. (a) MMP-9 was detected in a small number of epithelial cells at the pushing border in keratoacanthomas. (b) Expression of MMP-9 at the invasion front of SCC. (c) MMP-2 was not detected in keratinocytes, but was abundantly expressed in fibroblasts in all keratoacanthomas. (d) MMP-19 was detected at the epithelial pushing border in almost all keratoacanthomas. (e, f) MMP-19 disappears from the invasive cancer cell nests. (f) Higher magnification of the region in (e). Scale bars: 125 μ m (a, b, e), 50 μ m (c, d, f).

Figure 3 Expression of MMP-10, p16, and laminin-5 in keratoacanthoma. (a) Epithelial MMP-10 expression in keratoacanthoma. (b) Expression of MMP-10 in SCC. (c) Immunohistochemical staining for p16 in proliferating areas of keratoacanthoma. (d) In SCCs, p16 is mostly absent from the invasive front. The number of keratinocytes positive for laminin-5 γ 2 was higher in MMP-13-positive (e) than -negative (f) tumors. Scale bars: 125 μ m (a, c, d), 50 μ m (b, e, f).

protein was not generally expressed in keratinocytes. However, MMP-13 expression, a specific marker for malignant transformation in keratinocytes,^{36,37} was detected in the epithelium of half of the keratoacanthomas studied. Thus, staining for MMP-13 protein does not assist in distinguishing keratoacanthomas from grade I SCCs²⁶ and suggests that at least a subset of keratoacanthomas cannot be classified as benign. Low expression of another collagenase, MMP-8, was found in the epithelium in three of the keratoacanthoma samples, whereas neutrophils stained positively in the majority of specimens. In contrast to sporadic epithelial expression of MMP-8 in oral SCCs,³¹ cancer cells were devoid of this MMP in our cutaneous SCCs.

Transformed, but also normal migrating keratinocytes, express MMP-9,³⁸ which was detected in only 5/31 keratoacanthomas in keratinocytes (Table 2). Interestingly, Stevenson *et al*³⁹ have very recently shown in an EBV latent membrane protein 1 mouse model that with increasing epithelial hyperplasia and vascularization leading to keratoacanthoma, p16 and MMP-9 expression is induced. Expression of another gelatinase, MMP-2, was absent from the epithelial compartment of keratoacanthomas and SCCs. There exist contradictory reports on MMP-2 expression in cutaneous SCCs: using different antibodies than ours, Fundyler *et al*³² reported positive MMP-2 staining in keratinocytes of a subset of SCCs, but supporting our data, Tsukifugi *et al*⁴⁰ found positive MMP-2 staining only in fibroblasts surrounding SCCs.

MMP-10 protein was found in the epithelium of 28/31 keratoacanthomas and all SCCs. Based on our previous results in chronic wounds and SCC,⁴¹ expression of MMP-10 does not serve as a marker of invasion, but rather reflects inflammation and matrix remodeling associated with tumor growth. Appropriately regulated and localized expression of MMP-10 in keratinocytes is required for organized migration to the wound site and in that setting laminin-5 is the most important *in vivo* substrate of MMP-10.⁴²

MMP-19 was widely expressed by keratinocytes at the basal epidermal layer of keratoacanthomas and sporadically in 40% of the SCCs. We and others have previously reported that MMP-19 is absent from invasive cancer cell nests of well-differentiated SCC,^{14,34} and our new findings support these results. Thus, the loss of MMP-19 from basal epithelial cells of keratoacanthomas might serve as a warning sign of cancer development. In psoriasis, MMP-19 expression is associated with keratinocyte hyperproliferation⁴³ and for this reason it might also be upregulated in keratoacanthomas.

This is the first study on laminin-5 in keratoacanthoma. Cytoplasmic laminin-5 γ 2 chain serves as a marker of normal migrating wound keratinocytes and is a good indicator of migrating cancer cells in various tumor types as well.^{18,34} Several MMPs are known to process laminin-5 γ 2 *in vitro*.⁴⁴

We have previously shown that in cutaneous SCCs, laminin5 γ 2 is widely distributed along the invasive tumor front^{26,41} and thus our current data show that, in general, staining for laminin-5 cannot help in differentiation between keratoacanthoma and SCC. In this work, we found colocalization of laminin-5 and MMP-10, a phenomenon previously reported in squamous and basal cell cancers.⁴¹

In our keratoacanthoma samples, p16 was expressed by keratinocytes in the pushing border, although the staining intensity was variable among tumors (Table 1) perhaps owing to differences in UV exposure.⁴⁵ P16 expression is activated in cells in response to basement membrane degradation/invasion and is detected in normal migrating keratinocytes as well as in chronic wounds.^{11,27} Less than half of our SCC samples had occasional p16-positive keratinocytes at the tumor front. Others and we have shown previously that p16 is absent from invasive areas of SCCs¹⁷ and our new results support these findings.

This is the first comprehensive study on MMP expression in keratoacanthoma. Our novel results suggest that several MMPs are abundantly expressed in this tumor (Table 2). However, the presence of MMP-7 and -9 and the loss of MMP-19 and p16 should provoke suspicion of SCC. Our finding of the keratinocyte transformation-specific MMP-13 in lesions classified as keratoacanthomas support the idea that a subset of them are incomplete SCCs.

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