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ORIGINAL PAPER

Enhanced expression of nidogen 1 around the nest of basal cell carcinoma compared with that around squamous cell carcinoma

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Abstract

Basal cell carcinoma (BCC) is a malignant skin tumor originating from cells of the epidermal basal layer and adnexal epithelium, especially in sun-exposed areas. Unlike squamous cell carcinoma (SCC), BCC has a propensity to grow only locally possibly due to differences in the surrounding microenvironment including the basement membrane (BM) and stroma. To investigate the components constituting the BM and surrounding connective tissue in BCC and SCC, we analyzed the expression of BM proteins, nidogen 1 (NID1) and type IV collagen (COL4). We compared the immunohistochemical expressions of NID1 and COL4 among tumor specimens from BCC, SCC and its precancerous condition, actinic keratosis (AK), ($n=5$ each condition). The expressions of NID1 and COL4 were both decreased around the tumor nest of SCC. In contrast, the expressions of both NID1 and COL4 around the nest of BCC were much higher than in the peri-lesional normal skin not only at the BM, but also in the surrounding stromal tissue. Our findings imply that the surrounding stromal cells of BCC, but not SCC or AK, excessively produce NID1 and COL4, which may be involved in preventing BCC cells from destroying the BM and invading the dermis.

Keywords Basal cell carcinoma · Basement membrane · Cancer microenvironment · Nidogen 1 · Squamous cell carcinoma · Type IV collagen

Introduction

Recent epidemiological studies have shown that a growing number of individuals, along with the aging of the population, suffer from malignant skin tumors, including squamous cell carcinoma (SCC), basal cell carcinoma (BCC), malignant melanoma (MM) and actinic keratosis (AK), a precancerous lesion of SCC, particularly in the sun-exposed areas of the skin of older individuals [1, 2].

BCC is the most common cutaneous tumor worldwide. The number of BCC patients has been growing with the increasing numbers of people who take part in outdoor activities, as well as the aging of the society. In contrast to SCC and MM, both of which have metastatic ability, BCC usually remains locally, thus it rarely results in remote metastasis or patient's death [3].

The invasiveness and metastatic phenotype of the malignant skin tumors are known to depend on the conditions of the basement membrane (BM), which provides the surrounding cutaneous and vascular tissues with physical barriers and prevents the tumor cells from disseminating systemically [4].

The BM is a thin layer of highly specialized extracellular matrix (ECM), that underlies epithelial cells and separates these cells from and connects them to their interstitial matrix [5]. The main components of the BM are type IV collagen (COL4), laminins, perlecan and nidogens [6]. While the roles of some components have been studied in detail, the role of nidogens in the physiological and pathological skin conditions remains unclear.

Nidogens are linker proteins that connect to COL4 and laminin networks to stabilize the three-dimensional structure of the BM. The nidogen family in humans consists of two members; nidogen 1 (NID1) and nidogen 2 (NID2). NID1 is broadly found in the BM of most tissue types, while NID2

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is typically enriched in endothelial BM. Unlike other ECM molecules such as COL4, laminins and perlecan, which are derived from epidermal cells, the nidogens are derived from stromal fibroblasts [7, 8].

To our knowledge, the detailed roles of the BM components, in the skin tumor biology have been relatively studied well only on COL4, although the surrounding microenvironment including the BM and stroma in the tumor are known to be pivotal for tumor proliferation [9]. We therefore focused in the present study on assessing the expression of NID1 in the various cutaneous malignant conditions, mainly in the sun-exposed areas. We evaluated the change in the immunohistochemical expressions of the NID1 as well as COL4 as a reference in the tissue specimen from patients with AK, SCC and BCC.

Materials and methods

Patients

Paraffin-embedded tumor samples obtained from 15 total patients with AK (4 hypertrophic and 1 atrophic), SCC (5 well-differentiated SCC) and BCC (3 nodular BCC and 2 superficial BCC) between February 2016 and April 2017 were included in the present study. In addition, the normal skin surrounding each tumor tissue was used as a control. The clinical characteristics of the patients are summarized in Table 1. All of the tumors were solitary and well circumscribed.

The protocol of the present study was approved by the institutional review board at Osaka Medical College and the study was conducted according to the Declaration of Helsinki principles.

Table 1 Characteristics of the patients with AK, SCC and BCC in the present study

Patient no.	Age	Sex	Region	Histopathological type
AK (1)	93	F	Cheek	Hyperkeratosis
AK (2)	84	M	Cheek	Hyperkeratosis
AK (3)	56	F	Cheek	Atrophy
AK (4)	83	M	Temporal head	Hyperkeratosis
AK (5)	67	F	Cheek	Hyperkeratosis
SCC (1)	78	M	Dorsum of hand	Well-differentiated
SCC (2)	74	M	Abdomen	Well-differentiated
SCC (3)	75	F	Dorsum of foot	Well-differentiated
SCC (4)	100	F	Cheek	Well-differentiated
SCC (5)	88	M	Nose	Well-differentiated
BCC (1)	57	F	Axilla	Solid
BCC (2)	49	F	Lower eyelid	Superficial
BCC (3)	69	F	Cheek	Solid
BCC (4)	73	F	Nose	Superficial
BCC (5)	41	F	Parietal head	Solid

Primary and secondary antibodies

Commercially available primary antibodies were used for immunohistochemical analyses. Anti-human nidogen 1/entactin affinity purified polyclonal antibody AF25700-SP was obtained from R&D SYSTEMS (Minneapolis, MN, USA) and anti-human type IV collagen mouse monoclonal antibody (PHM-12, code: 413741) was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). A Histofine Simple Stain MAX-PO (MULTI) kit and MAX-PO(G) kit were also purchased from Nichirei Biosciences Inc. (Tokyo, Japan).

Immunohistochemistry

Formalin-fixed skin sections from five patients with AK, SCC and BCC each were subjected to immunohistochemical staining for NID1 and COL4 protein. For antigen retrieval, deparaffinized sections were kept in a staining trough filled with protease (415231, Nichirei Bioscience, Tokyo, Japan) for 15 min. The sections were then rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4) and exposed to peroxide blocking for 10 min at room temperature. The sections were then incubated with primary antibodies against NID1 and COL4 protein for 1 h. After rinsing twice with PBS, the sections were incubated for 30 min with secondary antibodies, which were conjugated with peroxidase-labeled dextran polymers. After rinsing again with PBS, the sections were treated with 3, 3′-diaminobenzidine as the substrate for peroxidase for 5 min. Finally, the sections were counterstained with hematoxylin, dehydrated and cleared in xylene prior to mounting in DPX (Entellan New, code: 107961, Merck, Kenilworth, New Jersey, USA). Negative controls were prepared by replacing the primary antibodies with PBS. The expression of NID1 and COL4 in the peri-lesional skin was compared with those in each tumor. Immunostaining images were acquired with an ECLIPSE 80i microscope (Nikon, Tokyo, Japan) and the Image J software program (NIH) was used for quantification in the co-localization analysis.

Statistical analyses

All quantitative values were expressed as the means ± standard deviation (SD) of the expressions in the tumor or control samples. Statistical significance between the tumor and control groups was evaluated using a Student's *t* test. Values of $p < 0.05$ were considered to be statistically significant.

Results

NID1 and COL4 expression in peri-lesional normal skin

Immunohistochemical studies of peri-lesional normal skin showed continuous staining of NID1 and COL4 in the BM at the dermal epidermal junction (Figs. 1a, c, 2a, c, 3a, c, arrowheads) as well as around blood vessels, sweat glands, hair follicles and fat cells. The expressions of NID1 and COL4 were the concurrent presence in linear and continuous deposition in all of the BM areas in the peri-lesional normal skin around SCC and BCC (Figs. 1a, c, 2a, c, 3a, c). No marked differences in the expression of NID1 or COL4 were noted between sun-exposed and non-sun-exposed areas (data not shown).

NID1 and COL4 expression in AKs

Immunostaining for both NID1 and COL4 in dysplastic areas containing BM in AK samples was continuous (Fig. 1b, d, arrowheads) and the intensities were slightly lower than in peri-lesional skin (Fig. 1a, c, arrowheads). This observation was not related to the histopathological features of AK (hypertrophic or atrophic) (data not shown). A quantitative analysis of the immunostaining signals appeared to show a slight decrease in the expressions of AK compared with those in the peri-lesional normal skin (Fig. 4a).

NID1 and COL4 expression in SCC

The intensity of staining for both NID1 and COL4 in dysplastic areas containing BM was discontinuous or partially absent and significantly lower than in peri-lesional skin (Fig. 2a–d). The expression of NID1 and COL4 in SCC samples showed a reduction in both NID1 and COL4 staining compared with that in peri-lesional skin samples. This observation was not related to the site location of SCC (sun-exposed or non-sun exposed) (data not shown). A quantitative analysis of the immunostaining signals showed that both NID1 and COL4 in SCC were significantly decreased compared with those in peri-lesional skin ($p < 0.05$) (Fig. 4b).

NID1 and COL4 expression in BCC

All five BCC samples showed continuous linear staining of BM around tumor islands for both NID1 and COL4. The intensity of staining for both NID1 and COL4 in dysplastic areas containing BM was continuous and increased compared to those in peri-lesional skin. Furthermore, the immunoreactivities for both NID1 and COL4 in all BCC samples were remarkably higher on focal staining than in peri-lesional skin, not only at the BM but also in the surrounding

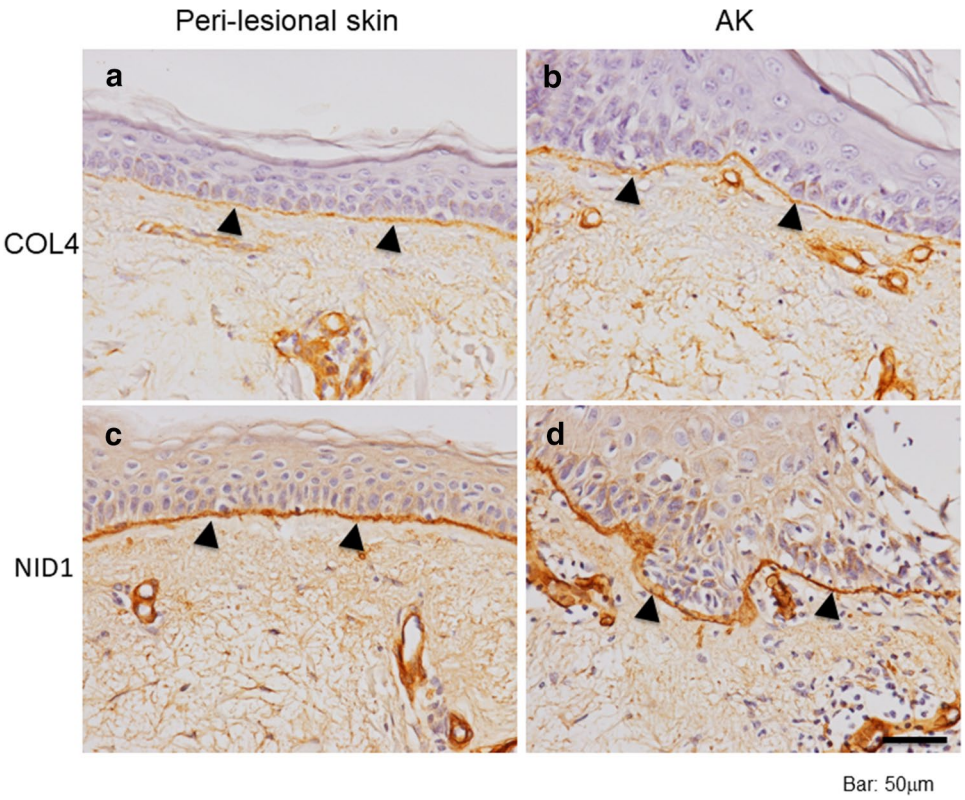

Fig. 1 The expressions of nidogen 1 and type IV collagen in AK. The expressions of COL4 and NID1 in epithelial BM were clearly visualized and found to be continuous in peri-lesional normal skin samples (**a, c**). Note that the expressions of both COL4 and NID1 in the BM of AK were continuous and slightly decreased compared with peri-lesional normal skin (**b, d**). Arrowheads indicate the BM at the dermal epidermal junction. Scale bar = 50 μm

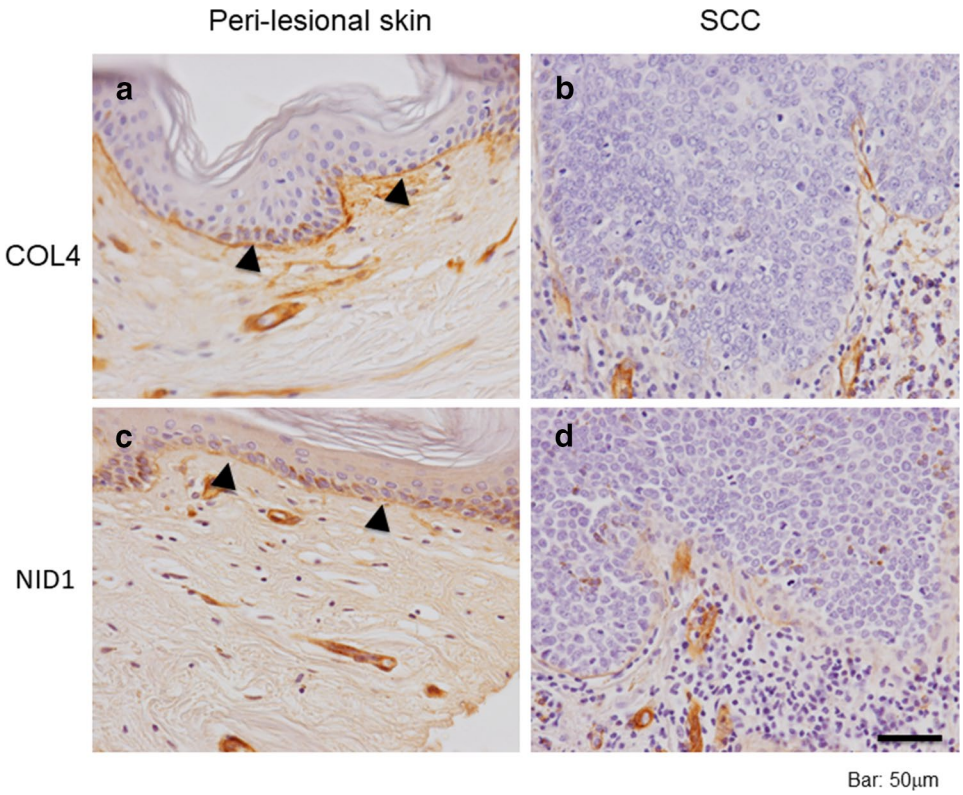

Fig. 2 The expressions of nidogen 1 and type IV collagen in SCC. Immunohistochemical localization of both COL4 and NID1 around SCC were discontinuous and significantly decreased (**b, d**) compared with that in the peri-lesional normal skin (**a, c**). Arrowheads indicate the BM at the dermal epidermal junction. Scale bar = 50 μm

stromal tissue (Fig. 3b, d, f, h). These findings were more obvious in nodular BCC (Fig. 3b, d) than in superficial BCC (Fig. 3f, h). This observation was not related to the site location (sun-exposed or non-sun- exposed) (data not shown). In addition, marked differences were noted in BCC compared with AK and SCC, as neither NID1 nor COL4 reacted to the stromal tissue (Figs. 1, 2b, d). A quantitative analysis of the immunostaining signals confirmed that both NID1 and COL4 were significantly increased in BCC compared with those in peri-lesional skin ($p < 0.05$) (Fig. 4c).

Discussion

In this study, we described for the first time the expression of NID1 in cutaneous malignant tumors such as AK, SCC and BCC as well as peri-lesional skin, although several reports have described the expression of COL4 in these conditions. Immunohistochemical studies demonstrated the expression of NID1 and COL4, which were concurrently present in the BM as linear and continuous deposition in AK, SCC and BCC. The expression of NID1 and COL4 were found to be decreased in SCC but not in AK compared with peri-lesional skin.

It has been reported that matrix metalloproteinase-2 (MMP-2) exerts degradative activity against components of the BM and may be involved in the progression of AK and SCC [10, 11]. MMP-2, which is activated by tumor cells of SCC, may degrade NID1 and COL4. Given that the intensity of NID1 and COL4 expression was significantly decreased in SCC, but not AK, the grade of tumor progression may be associated with the MMP-2-related degradation of BM proteins. In contrast, the expression of both NID1 and COL4 in BCC was much higher than in the peri-lesional skin, not only at the BM but also in the surrounding stromal tissue. This suggests that the BM surrounding BCC may be resistant to degradation unlike AK and SCC.

The BM in BCC is reported to be incomplete compared to that of normal skin [12, 13]. To investigate the changes in the property of BM in BCC, we focused on the expression of NID1, a major component of the BM, derived from stromal fibroblasts. Many electron microscopic studies have reported that the components of BM in BCC is relatively immature compared with those of normal human skin [12, 14–16, 20]. These studies have revealed the presence of poorly organized hemidesmosomes, discontinuous lamina densa, and a lack of anchoring fibrils. Other immunohistochemical studies have also reported the characteristic lack of bullous pemphigoid (BP) antigen [17, 18], some integrin components and type VII collagen as well as the presence of granular deposits of COL4 around the tumor cell nests of BCC [19–22].

It is becoming increasingly clear that BM and stromal microenvironments play a critical role in tumor development and progression [23–25]. However, the interactions between

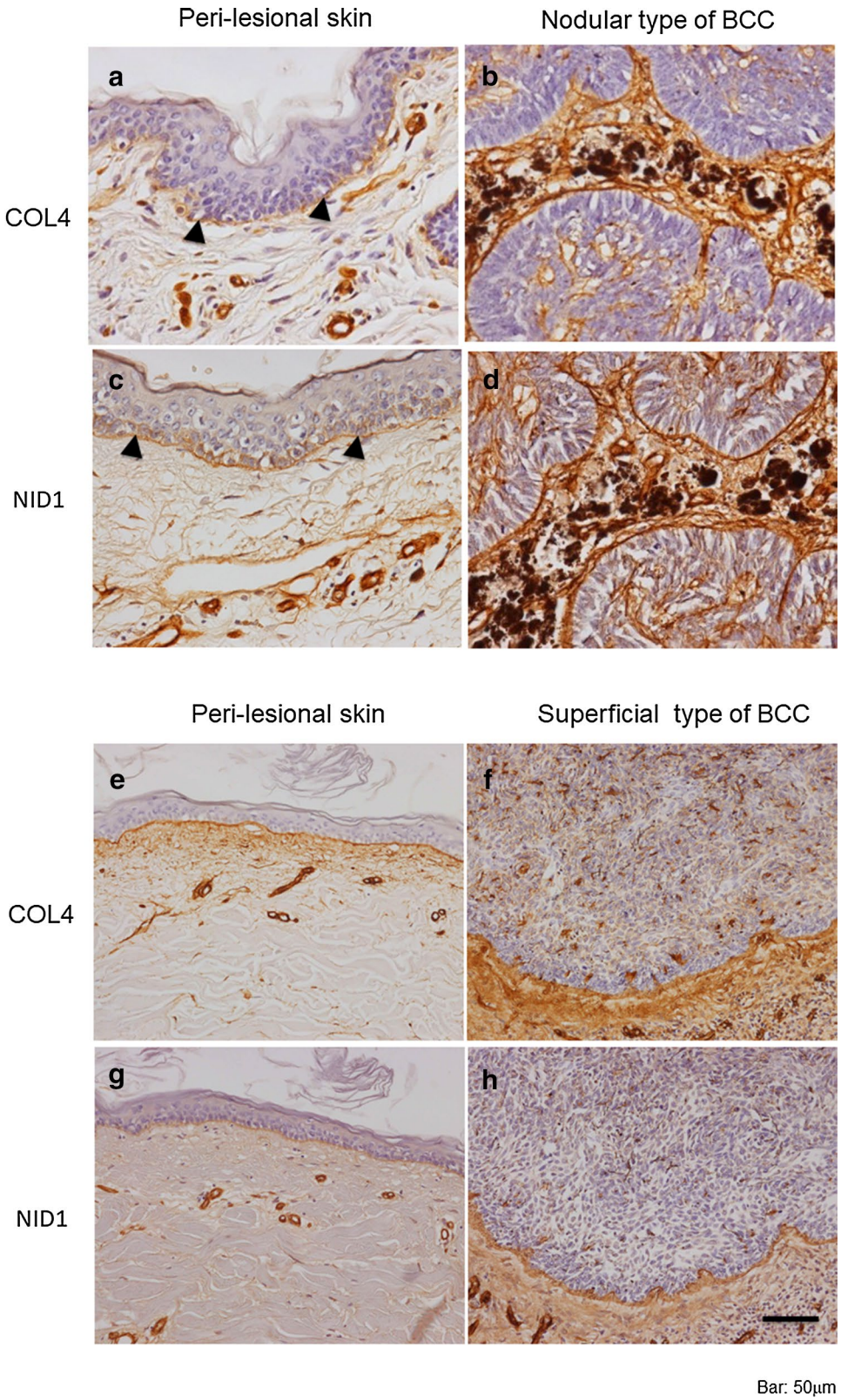

Fig. 3 The expressions of nidogen 1 and type IV collagen in BCC. Intense immunoreactivity for COL4 and NID1 was observed in the endothelial BM of the peri-lesional normal skin (**a, c, e, g**). The expressions of both COL4 and NID1 in nodular-type BCC were much higher than in peri-lesional normal skin not only at the BM but also in the surrounding stromal tissue (**b, d**). The expressions of both COL4 and NID1 in superficial-type BCC were also higher than in those of peri-lesional normal skin (**f, h**). Arrowheads indicate the BM at the dermal epidermal junction. Scale bar = 50 μm

the BM and BCC have been considered critical in local invasion of the tumors, as they depend on the production of degradative enzymes specific for the BM and surrounding connective tissue components. Tumor cells [26], including BCC [27, 28], produce collagenases, which digest collagen in the BM and surrounding stroma. Markey et al. reported

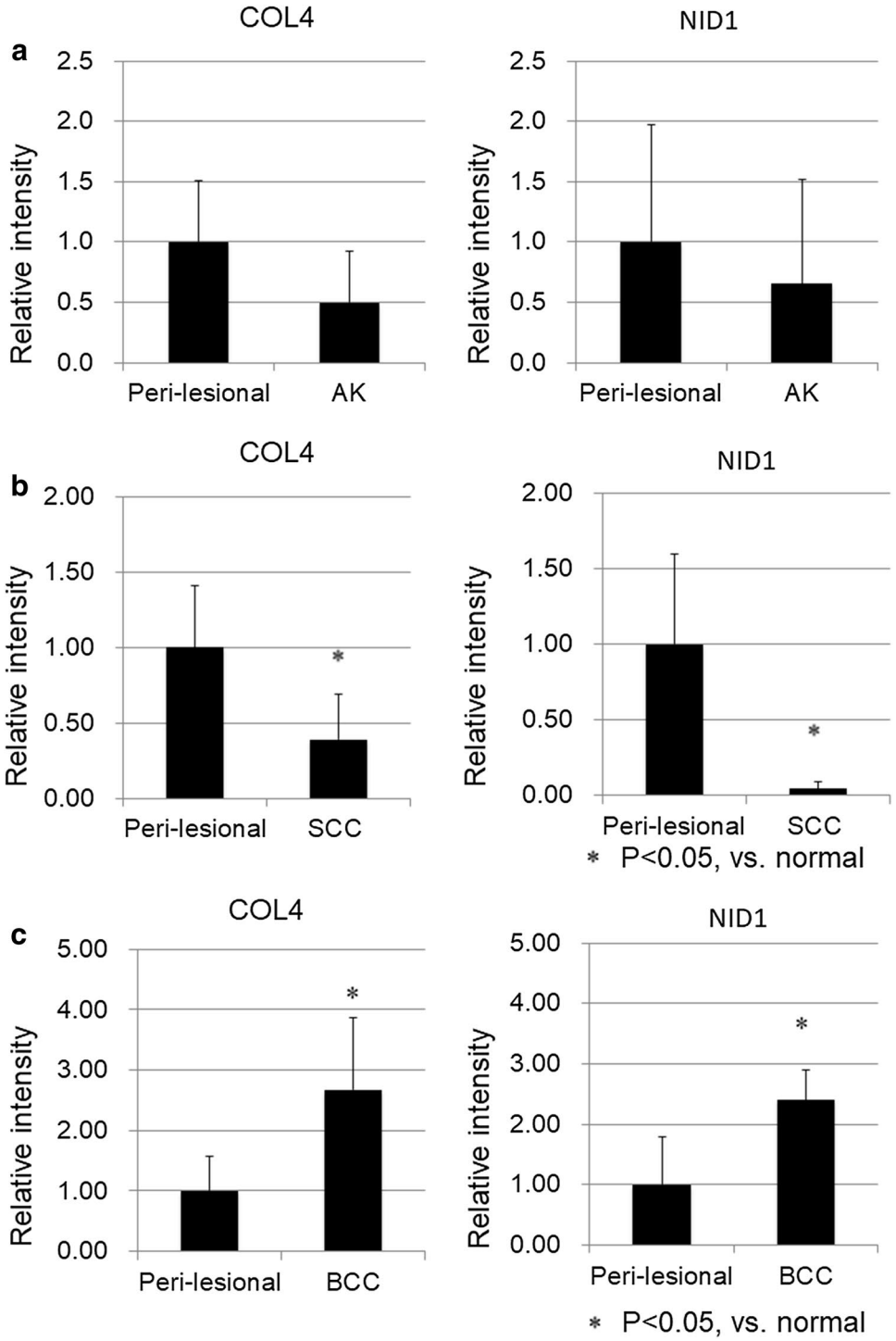

Fig. 4 Quantitative analyses of the expressions of nidogen 1 and type IV collagen in AK, SCC and BCC. Quantified immunostaining signals of COL4 and NID1 in AK ($n=5$) (**a**), SCC ($n=5$) (**b**) and BCC ($n=5$, 3 nodular type of BCCs and 2 superficial type of BCCs) (**c**) compared with those in the peri-lesional normal skin as the quantitative control. All values are shown as the means ± SD from 5 specimens of each tumor. *$p<0.05$ by a Student's *t* test

that the production of molecules such as collagenase by stromal cells, perhaps in response to factors released by tumor cells, leads to the selective dissolution of type VII collagen [21].

In the present study, we found that the expression of NID1 was not only present, but also increased in the BM and stromal tissues of BCC. Its expression pattern was parallel with that of COL4. One possible mechanism for the high expression of NID1 and COL4 in BCC is that the surrounding stromal cells of BCC start to produce BM components, including NID1 and COL4 in response to the growth of BCC. The excessive BM components may strengthen the BM around the tumor nest against the degradation of the tumor cells and only sequester the tumor at the primary site. In our observation, stromal fibroblasts in the peri-lesional normal skin showed faint NID1 immunostaining, while the

intensity of NID1 immunostaining increased in the stromal tissue around the BCC nests, implying that the fibroblasts surrounding the BCC nest might strongly express NID1. Quail and Joyce reported that cancer-associated fibroblasts (CAFs) play a critical role in tumor development and progression in invasive malignant tumors [25]. It is possible that CAFs that appear around the BCC nests (but not SCC nests) start to produce large amounts of NID1, which may play a role in keeping BCC localized, unlike SCC. Our data imply that NID1 and other BM proteins are involved in the suppression of BCC cells' infiltrative characteristics, making BCC only locally invasive and rarely expressing a metastatic phenotype.

Taken together, this study suggests that NID1 as well as COL4 may play a critical role in the tumor development of AK, SCC and BCC. The molecular role of NID1 and other factors related to the BM in the progression of BCC and other skin cancers should be elucidated in the future.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest in association with this study.

References

1. Diepgen TL, Mahler V (2002) The epidemiology of skin cancer. Br J Dermatol 146:1–5
2. Rigel DS (2008) Cutaneous ultraviolet exposure and its relationship to the development of skin cancer. J Am Acad of Dermatol 58:129–132
3. Marzuka AG, Book SE (2015) Basal cell carcinoma: pathogenesis, epidemiology, clinical features, diagnosis, histopathology, and management. Yale J Biol Med 88:167–179
4. Yurchenco PD (2011) Basement membranes: cell scaffoldings and signaling platforms. Cold Spring Harb Perspect Biol 3:a004911
5. Kruegel J, Miosge N (2010) Basement membrane components are key players in specialized extracellular matrices. Cell Mol Life Sci 67:2879–2895
6. Timpl R, Brown JC (1996) Supramolecular assembly of basement membranes. BioEssays 18:123–132
7. Chung AE, Durkin ME (1990) Entactin: structure and function. Am J Respir Cell Mol Biol 3:275–282
8. Gu H, Huang L, Wong YP, Burd A (2010) HA modulation of epidermal morphogenesis in an organotypic keratinocyte co-culture model. Exp Dermatol 19:e336–e339
9. Martino-Echarri E, Fernández-Rodríguez R, Rodríguez-Baena FJ, Barrientos-Durán A, Torres-Collado AX, Plaza-Calonge Mdel C, Amador-Cubero S, Cortés J, Reynolds LE, Hodivala-Dilke KM, Rodríguez-Manzaneque JC (2013). Contribution of ADAMTS1 as a tumor suppressor gene in human breast carcinoma. Linking its tumor inhibitory properties to its proteolytic activity on nidogen-1 and nidogen-2. Int.J.Cancer 33:2315–2324
10. Bhawan J (2007) Squamous cell carcinoma in situ of the skin: What does it mean? J Cutan Pathol 34:953–955
11. Fundyler O, Khanna M, Smoller BR (2004) Metalloproteinase-2 expression correlates with aggressiveness of cutaneous squamous cell carcinomas. Mod Pathol 17:496–502
12. Hashimoto K, Yamanishi Y, Dabbous MK (1972) Electron microscopic observations of collagenolytic activity of basal cell epithelioma of the skin in vivo and in vitro. Cancer Res 32:2561–2567
13. Mc Nutt NS (1976) Ultrastructural comparison of the interface between epithelium and stroma in basal cell carcinoma and control human skin. Lab Invest 35:132–142
14. Sonnenberg A, Calafat J, Daams H, van der Raaij-Helmer LM, Falcioni R, Kennel SJ, Aplin JD, Baker J, Loizidou M (1991) Integrin alpha 6/beta4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell - basement membrane adhesion. JCB 113:907–917
15. Zelickson AS (1968) An electron microscope study of the basal cell epithelioma. J Invest Dermatol 51:63–80
16. Kore-eda S, Matsuyoshi N, Ueda M, Horiguchi Y, Miyachi Y, Imamura S (2002) A monoclonal antibody established from the immunization of basal cell carcinoma (BCC) tissues reacts to the intercellular space of BCC and hair follicles. J Dermatol 29:728–725
17. Stanley JR, Beckwith JB, Fuller RP, Katz SI (1982) A specific antigenic defect of the basement membrane is found in basal cell carcinoma but not in other epidermal tumors. Cancer 50:1486–1490
18. Fairley JA, Heintz PW, Neuburg M, Diaz LA, Giudice GJ (1995) Expression pattern of the bullous pemphigoid-180 antigen in normal and neoplastic epithelia. Br J Dermatol 133:385–391
19. Weber L, Krieg T, Müller PK, Kirsch E, Timpl R (1982) Immunofluorescent localization of type IV collagen and laminin in human skin and its application in junctional zone pathology. Br J Dermatol 106:267–273
20. Van Cauwenberge D, Pierard GE, Foidart JM, Lapiere CM (1983) Immunohistochemical localization of laminin, type IV and type V collagen in basal cell carcinoma. Br J Dermatol 108:163–170
21. Markey AC, Tidman MJ, Churchill LJ, Aplin JD, Leigh IM, Purkis P, MacDonald DM (1991) The epidermal basement membrane in basal cell carcinoma: an immunohistochemical study. Br J Dermatol 125:21–26
22. Jones JCR, Steinman HK, Goldsmith BA (1989) Hemidesmosomes, collagen VII, and intermediate filaments in basal cell carcinoma. J Invest Dermatol 93:662–671
23. Liotta LA, Kleinerman J, Cantanzaro P, Rynbrandt D (1977) Degradation of basement membrane by murine tumour cells. J Natl Cancer Inst 58:1427–1431
24. Muller MM, Fusenig NE (2004) Friends or foes—bipolar effects of the tumour stroma in cancer. Nat Rec Cancer 4:839–849
25. Quail DF, Joyce JA (2013) Microenvironmental regulation of tumor progression metastasis. Nat Med 19:1423–1437
26. Dresden MH, Heilman SA, Schmidt JD (1972) Collagenolytic enzymes in human neoplasms. Cancer Res 32:993–996
27. Yamanishi Y, Dabbous MK, Hashimoto K (1972) Effect of collagenolytic activity in basal cell epithelioma of the skin on reconstituted collagen and physical properties and kinetics of the crude enzyme. Cancer Res 32:2551–2560
28. Barsky SH, Grossman DA, Bhuta S (1987) Desmoplastic basal cell carcinomas possess unique basement membrane-degrading properties. J Invest Dermatol 88:324–329