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Selective *in situ* protein expression profiles correlate with distinct phenotypes of basal cell carcinoma and squamous cell carcinoma of the skin

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Summary. Non-melanoma skin cancer is the most common malignancy that shows increasing incidence due to our cumulative exposure to ultraviolet irradiation. Its major subtypes, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) differ in pathobiology, phenotype and clinical behavior, which must be reflected at the molecular level. In this study, protein expression profiles of BCC and SCC were tested in tissue microarrays and correlated with that of actinic keratosis, Bowen's disease, seborrheic keratosis and normal epidermis by detecting 22 proteins involved in cell interactions, growth, cell cycle regulation or apoptosis. The significantly more reduced collagen XVII, CD44v6, pan-Desmoglein levels and more evident E-Cadherin delocalization in BCC compared to SCC correlated with the *de novo* dermal invasion of BCC against the progressive invasion from *in situ* lesions in SCC development. EGFR was also expressed at a significantly higher level in SCC than in BCC. The upregulated cell communication protein connexin43 in BCC could contribute to the protection of BCC from metastatic invasion. Elevated cell replication in BCC was underlined by the increased topoisomerase IIα and reduced $p21^{waf1}$ and $p27^{kip1}$ positive cells fractions compared to SCC. Compared to differentiated keratinocytes, caspase-8 and -9 were equally upregulated in skin carcinoma subtypes for either mediating apoptosis induction or immune escape of tumor cells. Hierarchical cluster analysis grouped SCC and actinic keratosis cases exclusively together in support of their common origin and malignant phenotype. BCC cases were also clustered fully together. Differentially expressed proteins reflect the distinct pathobiology of skin carcinoma subtypes and can serve as surrogate markers in doubtful cases.

Key words: Skin cancer, Squamous cell carcinoma, Basal cell carcinoma, Tissue microarray, Differential protein expression, Hierarchical cluster analysis

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Introduction

The incidence of skin cancer has been constantly rising in the white-skinned population worldwide, increasingly affecting younger age generations, possibly due to the cumulative lifetime exposure to ultraviolet (UV) irradiation (Christenson et al., 2005; Kutting and Drexler, 2010). Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the major subtypes of non-melanoma skin cancer, which is more common than the rest of all cancers together (Lomas et al., 2012). Though the majority of BCC and SCC can be diagnosed using H and E morphology, differential biomarker expression can underline their distinct pathobiology and help identify borderline cases (Boukamp, 2005). We have recently demonstrated that type XVII collagen, a hemidesmosomal adhesion protein of basal keratinocytes is upregulated in SCC as early as in actinic/solar keratosis (AktKer), but not expressed in BCC (Stelkovics et al., 2008). In this study using tissue microarrays (TMA), we further searched for selective

biomarker profiles in epidermal keratinocyte tumors with particular attention to the difference between BCC and SCC.

Despite the similar UV exposure associated genetic and epigenetic deviations in BCC and SCC, their diverse etiology is manifested by distinct phenotypes and molecular features (Tsai and Tsao, 2004). BCC is thought to develop *de novo* as a result of high dose intermittent sun exposure (Boukamp, 2005) and forms locally invasive nests (superficial, nodular, or micronodular) that very rarely metastasize (von Domarus and Stevens, 1984). On the contrary, SCC is known to develop through gradual progression from precancerous through *in situ* malignant lesions (AktKer, Bowen's disease) (Marks et al., 1988; Heaphy and Ackerman, 2000) mainly in association with chronic sun exposure (Boukamp, 2005) or oncogenic papilloma virus (HPV) induction (Moy et al., 1989). As opposed to BCC, SCC may form metastases in 0.5-16% of the cases with a 5-year survival rate of 25-50% (Cherpelis et al., 2002).

Several molecular pathways have been implicated in the development and progression of non-melanoma skin cancers (Boukamp, 2005; Greinert, 2009). These involve the deregulated cell cycle (Brown et al., 2004), apoptosis (Erb et al., 2005), cell growth (Uribe and Gonzalez, 2011), cell interactions (Papadavid et al., 2002) and communication (Haass et al., 2006) in co-operation with impaired immune response (Rangwala and Tsai, 2011), resulting in keratinocyte transformation, uncontrolled cell cycle progression and carcinoma invasion. However, the studies testing these pathways at the level of gene and protein expression have focused on either a limited number of cases or biomarkers (Wrone-Smith et al., 1999). Furthermore, they occasionally led to contradicting results, possibly due to applying diverse immunostaining techniques in different patient cohorts.

TMAs support high throughput expression profiling under standard conditions i.e. the testing of a series of cancer related proteins in many tissue samples within reasonable time (Kononen et al., 1998). Here we detected a range of proteins involved in the regulation of keratinocyte interactions (connexin43 - Cx43, pan-Desmoglein, E-Cadherin, ß-Catenin, CD44v6), basal membrane anchorage (collagen XVII), cell proliferation (Ki67, cyclin D1, cyclin D3, Topoisomerase IIα - TopoIIa), cell cycle control ($p16^{ink4}$, $p21^{waf1}$, $p27^{kip1}$, p53, p63), growth promotion (EGFR, Akt) and apoptosis (caspase-8, caspase-9 and caspase-3) in TMA samples of BCC, SCC, AktKer, seborrheic keratosis (SebKer) and normal epidermis to reveal correlations and selective protein expression profiles that can also be exploited for diagnostic purposes.

Materials and methods

Surgical skin biopsy samples and tissue microarray construction

Tissue blocks of skin resection specimens fixed in 10 % formalin and embedded routinely in paraffin-wax were collected from the archives of the Department of Dermatology and Allergology, Faculty of Medicine, University of Szeged, Hungary. Histopathological diagnoses were made based on standard criteria (LeBoit et al., 2006). A total of 172 cases of benign and malignant skin samples, dated between 2001 and 2004, including 9 normal skin, 14 SebKer, 31 AktKer and primary carcinomas of 65 SCC (53 well to moderately, and 12 poorly differentiated), 16 BsCC and 37 nodular BCC types were tested. The mean age of the patients was 72 years (ranging from 34 to 101 years) with a sex ratio (M/F) of 78/94. The mean diameter of tumors was 23 mm (4-55 mm) for SCC; 12 mm (5-30 mm) BCC, and 15mm (7-20 mm) for BsCC. TMA blocks were constructed using a manual tissue arrayer (Histo-pathology Kft., Pecs, Hungary) as published earlier (Stelkovics et al., 2008). Tissue cores of 2 mm diameter were selected for punching under microscopic control of the relevant Hand E stained sections and incorporated into 8 pieces of 24-sample TMA blocks. In most cases the relatively large core diameter allowed the representation of the particular lesion by using a single core sample. In a few cases of significant tumor heterogeneity the donor samples were duplicated. All examinations in this study were performed under the ethical approval of Ethical Review Boards of the Medical Faculties both at the University of Szeged and Semmelweis University, Budapest, Hungary (KL-37/2006).

Immunohistochemistry

For immunohistochemistry 4 Ìm thick serial sections were cut from the TMA blocks. The sections were mounted on charged SuperFrost Ultra Plus glass slides (Menzel GmbH, Braunschweig, Germany) and heated overnight at 65°C to ensure firm section adhesion. Following routine dewaxing in 3 changes of xylene and rehydration in descending ethanol series endogenous peroxidases were quenched using 1 % hydrogen peroxide in methanol for 30 minutes. Heat-induced epitope retrieval (HIER) was performed by heating sections in a mixture of 0.1 M Tris-base and 0.01 M EDTA (pH 9.0) (Tris-EDTA) for 2 minutes in a pressure cooker (Tefal Clipso) at full pressure (~120°C). Specifications and dilutions of the primary antibodies and the scoring criteria of the immunostaining results are summarized in Table 1. All antibodies were commercially obtained except the monoclonal mouse antibody clone 9G2, which recognizes the aa507-527 sequence of collagen XVII protein (also known as BP180 or BPAG2) as characterized and published earlier (Stelkovics et al., 2008). For antibodies, which were not routinely used, immunostaining was first optimized by testing a series of their 2-fold dilutions in control tissues after standard Tris-EDTA antigen retrieval (see above) and immunodetection. The EnVision+ detection system (Dako, Glostrup, Denmark) was applied for 40 min following incubation for 90 min with the primary

antibodies. Finally, the specific peroxidase activity was revealed using a DAB (diaminobenzidine) hydrogen peroxide chromogen-substrate kit (Dako) for 3-8 min. The sections were washed between each incubation step using 0.1 M Tris-HCl (pH7.4) buffered saline (TBS). After counterstaining with hematoxylin, they were coverslip mounted using Faramount (Dako). Immunofluorescence detection was used to reveal punctuate Cx43 positive signal, which are often less than a 1 μm size, in combination with detecting Ki67 positive proliferating cells. Tissue bond rabbit Cx43 antibody was detected with Alexa546 (1:200, orange-red), mouse Ki67 IgG was revealed with Alexa 488 (1:200, green) and cell nuclei were stained with DAPI (all obtained from Invitrogen-Molecular Probes, Carlsbad, CA).

Scoring, statistics and hierarchical cluster analysis

Immunostained sections were digitalized using Pannoramic Scan (3DHISTECH, Budapest, Hungary) and scored by at least 2 assessors on a 4-scale system (0= negative; 1= week positive; 2= moderate positive and 3= strong positive) after setting up standard criteria for each marker (Table 1). Assessors involved were a consultant dermatopathologist (IK), a senior research scientist experienced in dermatology research (TK) and a PhD student (ES). Discordant scores were consolidated upon final agreement between assessors. In case of duplicate samples the higher score was always kept for further analysis. Thresholds were based on literature data and by considering the biological roles of the tested molecule. Therefore, adequate subcellular localization was critical at scoring, and immunoreactions not related directly to the epidermis at benign or *in situ* lesions or to the epidermal tumor component in malignant lesions were not assessed. Any immunostaining was considered positive when an obvious signal was seen in the right subcellular compartment.

For statistics the Pearson's chi-square (χ^2) and the Fisher exact tests of the SPSS, v15.0 software package (SPSS Co. Chicago, IL) were applied to see which biomarker discriminated significantly between SCC and BCC groups. Two separate thresholds were tested. In the first, cases scoring 0 and 1 were considered negative against those scoring 2 and 3 (positive). In the other, cases scoring 0 (negative) were correlated with those scoring 1-3 (positive). Since the first cutoff was more reproducible for all assessors involved, this was utilized for binary statistics. Significant difference was declared at p-values of <0.05.

For cluster analysis, immunostaining scores were converted into the relevant format using the TMA-Deconvoluter software (Liu et al., 2002). Unsupervised hierarchical (average linkage) clustering was done with the Cluster software and the results were graphically depicted using Treeview heat-maps (Eisen et al., 1998) including correlation coefficients calculated by the software for selected clusters. Both software tools are

Table 1. Antibody specifications and scoring criteria for the immunostaining results.

Antigen (clone/code)	Vendor (dilution)	Staining pattern	Score 1	Score 2 % positive cells	Score 3
Pan-Cytokeratin (AE1/AE3)	Dako (kit, 1:2)	CP	na	na	na
Pan-Cytokeratin (MNF116)	Dako (1:100)	CP	na	na	na
*Collagen XVII (9G2)	Home-made (1:50)	M/CP	5-15	15-50	>50
Connexin43 (#3512)	Cell Sign. 1:100	Punctate M/CP	10-20	20-40	>40
pan-Desmoglein (AHP321)	Serotec (1:3000)	M/CP	20-40	40- 70	>70
E-Cadherin (36B5)	LabVision (1:100)	M/CP	20-40	40-70	>70
ß-Catenin (17C2)	LabVision (1:50)	M/CP	20-40	40-70	>70
CD44v6 (VFF-7)	Novocastra (1:50)	M/CP	30-50	50-80	>80
EGFR (EGFR.25)	Novocastra (1:100)	M/CP	10-20	20-50	>50
pan-Akt (ab8805)	Abcam (1:100)	CP/N	20-40	40-70	>70
$p16^{link4}$ (6H12)	Novocastra (1:30)	N/CP	5-15	15- 40	>40
$p21^{waf1}$ (SX118)	Dako (1:50)	N	10-20	20-50	>50
$p27^{kip1}$ (SX53G8)	Dako (1:50)	N	15-30	30-50	>70
p53 (DO7)	Dako (kit, 1:2)	N	5-15	15-40	>40
p63 (4A4)	LabVision (1:400)	N	30-50	50-80	>80
Ki67 (Mib1)	Dako (kit, 1:2)	N	5-15	15-40	>40
Cyclin D1 (SP4) rabbit	LabVision (1:100)	N	5-15	15-40	>40
Cyclin D3 (DCS-22)	Dako (1:30)	N	10-20	20-50	>50
Topoisomerase IIα (3F6)	Novocastra (1:20)	N	5-15	15-30	>30
Caspase-3 (3CSP03)	LabVision (1:100)	CP/N	5-15	15-50	>50
Caspase-8 (RB-1200)	LabVision (1:200)	CP	10-20	20-60	>60
Caspase-9 (RB-1205)	LabVision (1:200)	CP/N	10-20	20-50	>50

Dako: Glostrup, Denmark; Cell Sign.: Cell Signaling, Danvers, MA, USA; Serotec: ADB Serotec, Kidlington, UK; LabVision: Thermo, Kalamazoo, MI, USA; NovoCastra: Leica, NewCastle, UK; CP: cytoplasmic; M: cell membrane; N: nuclear; na: not applicable; *: Characterised in Stekovics et al, 2008.

freely available at http://rana.ibi.gov/eisenSoftware.htm.

Results

Biomarker expression in normal epidermis

Fig. 1 demonstrates major expression patterns of the studied biomarkers in the epidermis of normal morphology or epidermal hyperplasia, as is also summarized in Table 2. Wide spectrum cytokeratin antibodies (AE1/AE3 and MNF116) used for identifying keratinocyte origin of skin lesions labeled all layers in normal epidermis. Proteins known to be functionally associated with keratinocyte membranes, including the cell adhesion molecules CD44v6, pan-Desmoglein and E-Cadherin, the microfilament anchoring ß-Catenin and the gap junction channel forming Cx43 showed increasing gradients towards the differentiated keratinocyte layers. Collagen XVII antibody distinctly labeled the undifferentiated basal keratinocytes where cell membrane EGFR expression was also the strongest. EGFR levels showed a decreasing gradient along keratinocyte differentiation. Akt reaction was seen in most epidermal keratinocytes, cytoplasmic in the upper

Table 2. Biomarker expression in epidermal keratinocyte tumors and normal epidermis with significant differences shown between relevant groups.

Protein detected	NormEpid Basal keratinocytes	NormEpid Differentiated keratinocytes	SebKer	ActKer	Bowen's	SCC	BsCC	BCC	P-value						
									DiffKer vs SCC	Diff/Ker vs BCC	SCC vs BCC	AktKer vs SCC	Bowen vs BCC	AktKer vs BCC	Bowen vs SCC
% Positive cases (positive/all cases)															
CollXVII (9G2)	100 (9/9)	0 (0/9)	14 (2/14)	57 (16/28)	63 (10/16)	97 (56/61)	33 (5/15)	6 (2/37)	<0.001*	ns	<0.001	<0.001*	<0.05*	<0.001	<0.001
Connexin43	11 (1/9)	100 (9/9)	100 (14/14)	92 (24/26)	75 (12/16)	32 (19/58)	50 (7/14)	76 (27/36)	<0.001 Delocal.	ns	<0.001*	<0.001	<0.05	<0.05*	ns
pan-Desmoglein	33 (3/9)	100 (9/9)	100 (14/14)	93 (25/27)	87 (13/15)	95 (57/60)	7 (1/14)	12 (4/37)	ns	<0.001	<0.001	ns	ns	<0.001	<0.001
E-Cadherin	22 (2/9)	100 (9/9)	100 (14/14)	89 (25/28)	81 (13/16)	74 (42/57)	47 (7/15)	43 (16/37)	ns	<0.001 Delocal.	<0.05 Delocal.	ns	ns	<0.001 Delocal.	<0.05 Delocal.
ß-Catenin	67 (6/9)	100 (9/9)	93 (13/14)	86 (25/29)	69 (11/16)	72 (44/61)	67 (10/15)	84 (29/35)	ns	ns	ns	ns	ns	ns	ns
CD44v6	100 (9/9)	100 (9/9)	93 (13/14)	100 (28/28)	87 (13/15)	95 (58/61)	67 (10/15)	53 (19/36)	ns	<0.05	<0.001	ns	ns	<0.001	<0.05
EGFR	100 (9/9)	44 (4/9)	43 (6/14)	83 (24/29)	81 (13/16)	86 (51/59)	69 (9/13)	62 (23/37)	<0.05*	ns	<0.05	ns	ns	ns	ns
pan-Akt	67 (6/9)	44 (4/9)	71 (10/14)	85 (23/27)	73 (11/15)	89 (54/61)	71 (10/14)	90 (32/36)	<0.05*	<0.001*	ns	ns	ns	ns	ns
$p16^{ink4}$	0 (0/9)	22 (2/9)	35 (5/14)	46 (13/28)	53 (8/15)	69 (40/58)	67 (10/15)	78 (28/36)	<0.05*	<0.001*	ns	<0.05*	ns	<0.005*	<0.05*
$p21^{waf1}$	22 (2/9)	44 (4/9)	50 (7/14)	78 (19/27)	69 (11/16)	83 (49/59)	40 (6/15)	57 (20/35)	<0.05*	ns	<0.05	ns	ns	ns	ns
$p27^{kip1}$	0 (0/9)	100 (9/9)	100 (14/14)	91 (26/29)	81 (13/16)	90 (55/61)	57 (8/14)	67 (24/36)	ns	<0.05	<0.05	ns	ns	<0.05	ns
p53	22 (2/9)	11 (1/9)	43 (6/14)	67 (18/27)	67 (10/15)	76 (45/59)	73 (11/15)	84 (31/37)	<0.001*	<0.001*	ns	ns	ns	ns	ns
p63	100 (9/9)	78 (7/9)	93 (13/14)	91 (26/29)	87 (13/15)	100 (60/60)	93 (13/14)	100 (36/36)	ns	ns	ns	ns	ns	ns	ns
Ki67	100 (9/9)	11 (1/9)	57 (8/14)	70 (19/27)	69 (11/16)	75 (45/60)	73 (11/15)	83 (30/36)	<0.001*	<0.001*	ns	ns	ns	ns	ns
Cyclin D_1 (SP4)	100 (9/9)	0 (0/9)	36 (5/14)	62 (18/29)	63 (10/16)	69 (42/61)	73 (11/15)	67 (23/34)	<0.001*	<0.001*	ns	ns	ns	ns	ns
Cyclin D3	56 (5/9)	44 (4/9)	50 (7/14)	67 (18/27)	69 (11/16)	79 (48/61)	67 (10/15)	69 (25/36)	<0.05*	ns	ns	ns	ns	ns	ns
Topoisomerase IIα	100 (9/9)	11 (1/9)	36 (5/14)	59 (17/29)	56 (9/16)	62 (37/60)	86 (12/14)	83 (29/35)	<0.05*	<0.001*	<0.05*	ns	ns	<0.05*	<0.05*
Caspase-8	89 (8/9)	33 (3/9)	86 (12/14)	83 (20/24)	67 (10/15)	63 (38/60)	60 (9/15)	74 (26/35)	<0.05*	<0.05*	ns	ns	ns	ns	ns
Caspase-9	78 (7/9)	22 (2/9)	71 (10/14)	81 (21/26)	56 (9/16)	68 (41/60)	71 (10/14)	65 (22/34)	<0.05*	<0.05*	ns	ns	ns	ns	ns
Caspase-3	78 (7/9)	56 (5/9)	57 (8/14)	63 (17/27)	56 (9/16)	49 (29/59)	40 (6/15)	57 (20/35)	ns	ns	ns	ns	ns	ns	ns

NormEpid: normal epidermis; SebKer: seborrheic keratosis; AktKer: actinic keratosis; Bowen's: Bowen's disease; SCC: squamous cell carcinoma; BCC: basal cell carcinoma; BsCC: basosquamous carcinoma. *: show the increasing tendency of expression in the second group comapared.

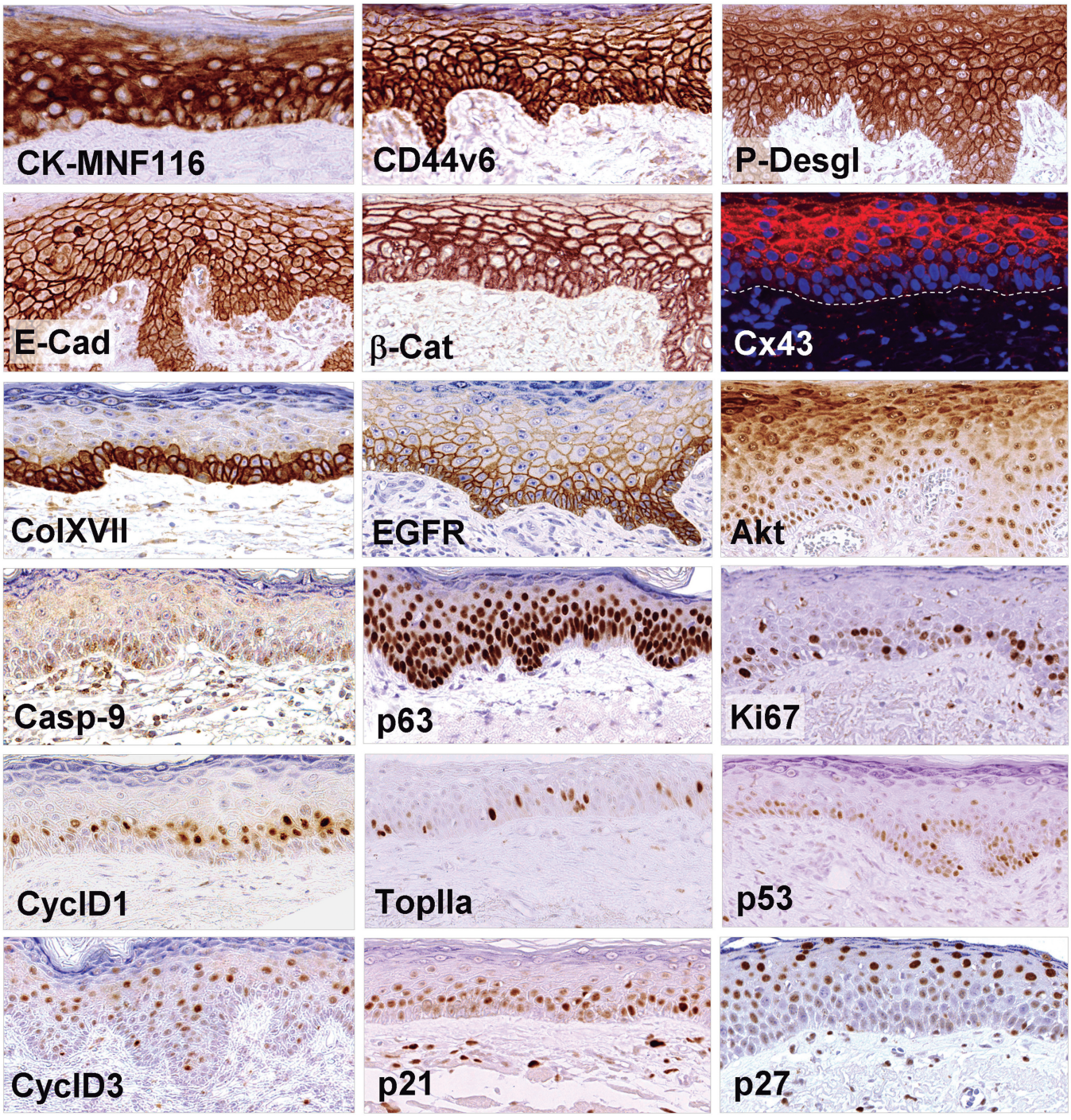

Fig. 1. Cellular and sub-cellular (cell membrane: m; cytoplasmic: cp; nuclear: n) localization of the tested biomarkers in the normal epidermis. Basal-suprabasal keratinocytes proliferate then gradually differentiate in the upper layers. Wide spectrum cytokeratin (CK-MNF116; cp), CD44v6 (m) and pan-desmoglein (P-Desgl; m, cp) reactions label all keratinocytes in normal epidermis. E-Cadherin (E-cad; m), ß-Catenin ß-Cat; m) and the connexin43 gap junction (Cx43; m) protein are concentrated more in the differentiated epidermis, while collagen XVII (ColXVII) is distinctly expressed in basal undifferentiated keratinocytes. EGFR (m, cp) production is inversely proportional with keratinocyte differentiation and Akt (cp, n) is more cytoplasmic in the differentiated layers and nuclear in the basal layer. Weak caspase-9 (Casp-9; cp) and strong p63 (n) signals are mainly localized to the undifferentiated keratinocytes. The nuclear regulators of cell replication Ki67, cyclin D1 (CyclD1) and topoisomerase IIα (TopIIa), along with weak p53 signal can be detected in the undifferentiated keratinocytes. Cyclin D3 (CyclD3) and the cyclin dependent kinase inhibitor $p21^{waf1}$ (p21) can be seen both in some basal and differentiated cells, while $p27^{kip1}$ (p27) is localized more to the differentiated epidermis. Immunoperoxidase reactions (brown), except that detecting Cx43, which is immunofluorescence (rodamine - red; DAPI - blue). x 200

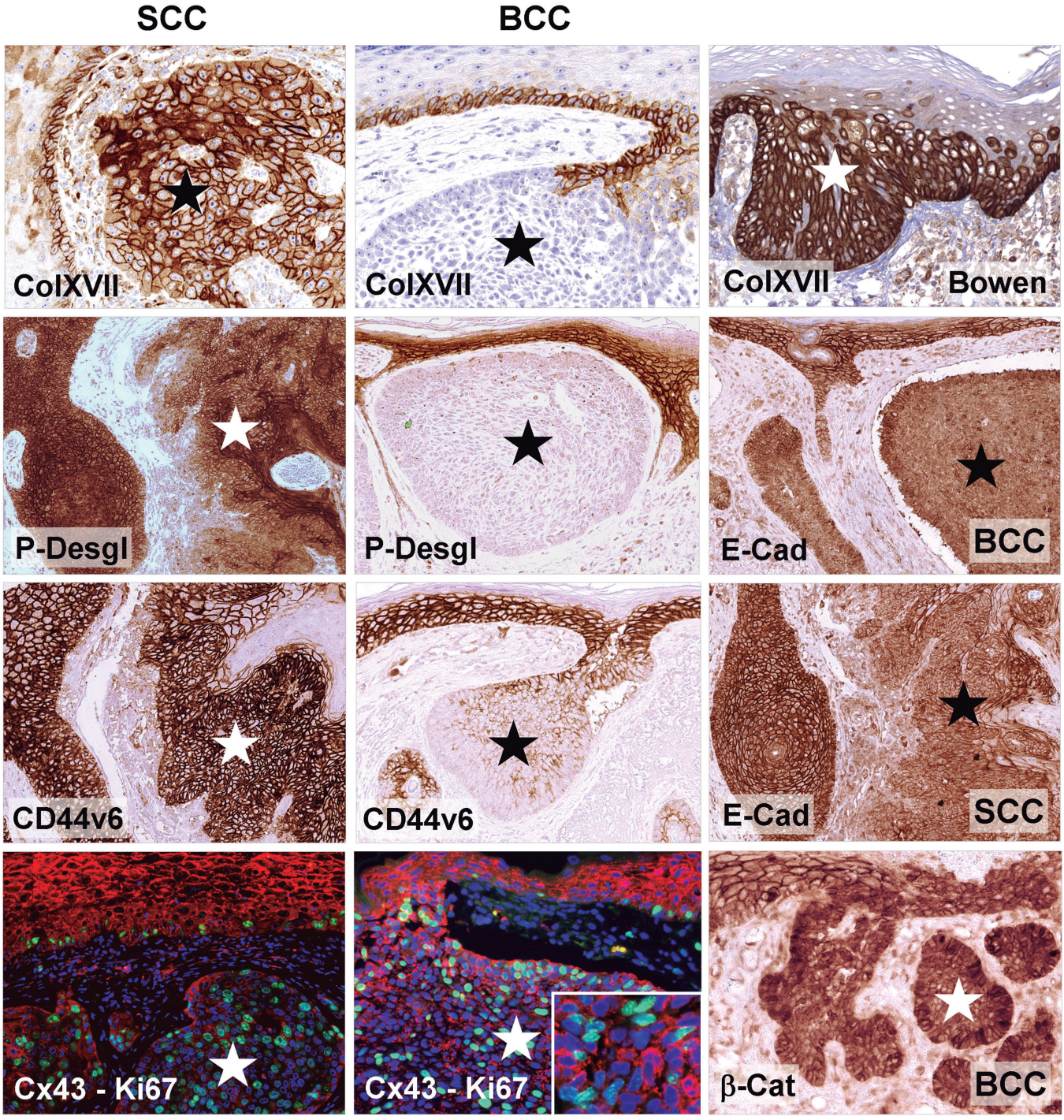

Fig. 2. Differential expression of cell-cell interaction proteins in non-melanoma skin cancers. Asterisks highlight representative tumor nests adjacent to normal epidermis or epidermal hyperplasia. In SCC (left column) collagen XVII (ColXVII), pan-desmoglein (P-Desgl) and CD44v6 proteins are produced at high levels, while they are downregulated in BCC (middle column). On the contrary, connexin43 (Cx43; red) is expressed at significantly lower level in SCC (bottom left) than in BCC (bottom middle). Strong proliferation in Cx43 stained tumors is revealed by nuclear Ki67 reaction (green) in both carcinoma subtypes. Atypical cells of Bowen's disease (Bowen) are also strongly ColXVII positive (uppermost right panel). E-Cadherin (E-Cad) is more delocalized to the cytoplasm in BCC than in SCC (right middle panels) despite their similar level of protein expression. Besides cytoplasmic delocalization ß-Catenin (ß-Cat) occasionally shows nuclear translocation in a BCC (bottom right panel). Immunoperoxidase reactions (brown), except those detecting Cx43 (rodamine, red) and Ki67 (FITC, green), which are immunofluorescence. 1st row, 4th row, x 200; 2nd row, 3rd row, x 100

and nuclear in the lower cell layers. Apoptosis related caspase-9 and caspase-3 were

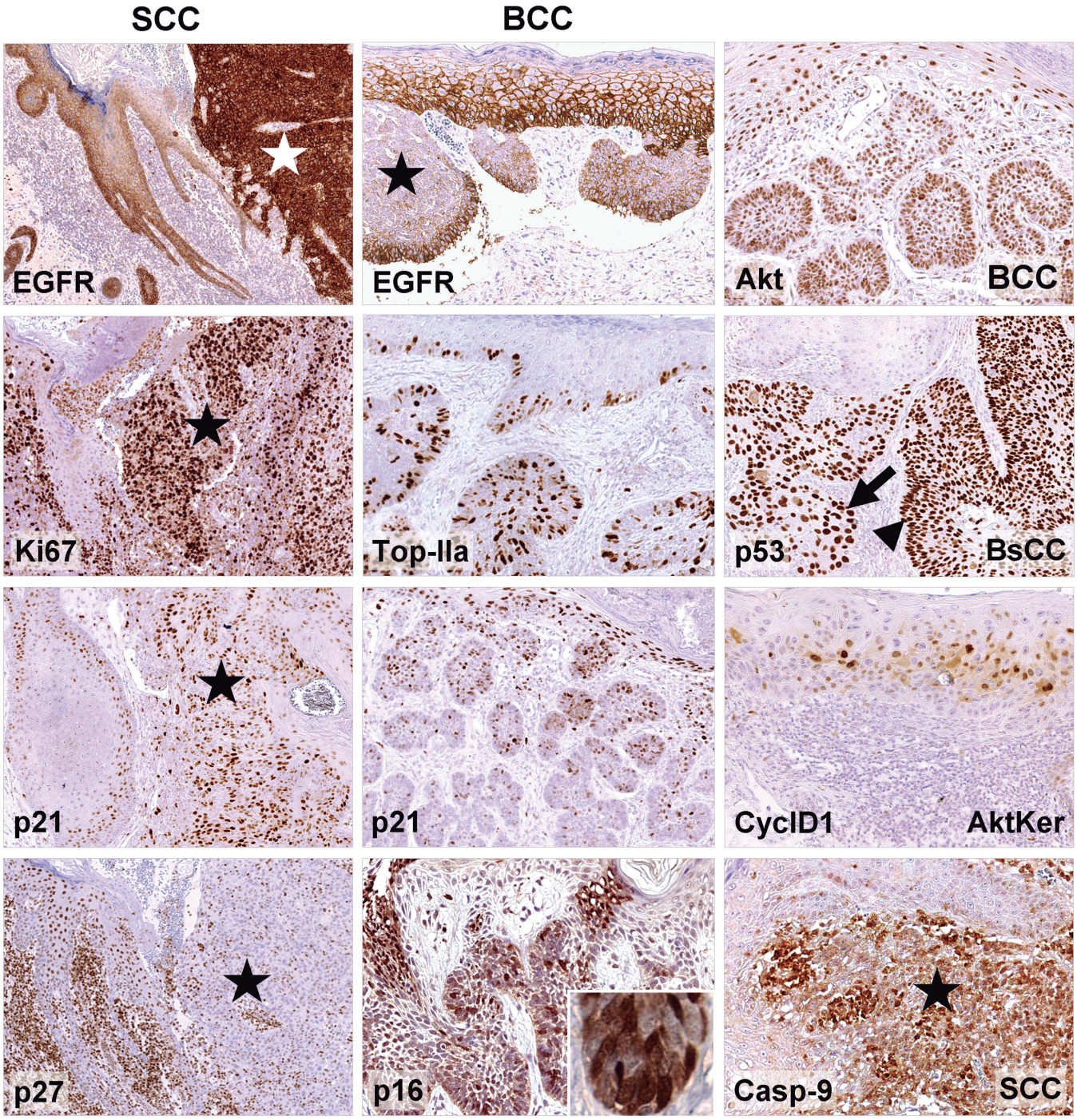

Fig. 3. Expression of proteins regulating cell growth and replication in non-melanoma skin cancers. Asterisks highlight tumor nests where they are not obvious. In serial sections of an SCC (left column) EGFR, Ki67 and $p21^{waf1}$ (p21) proteins are upregulated, while $p27^{kip1}$ (p27) protein is downregulated (asterisk) with only pre-existing epidermal cells and lymphocytes remaining positive. In BCC (middle column), reduced EGFR and elevated topoisomerase IIα (TopIIa) expression is seen compared to the normal epidermis and to differentiated keratinocytes, respectively. The number of $p21^{waf1}$ (p21) positive cells is also reduced in vertical tumor nests. Nuclear $p16^{ink4}$ (p16) positive cells are frequent in the tumor and focal in the epidermal lesion. Inset highlights cytoplasmic delocalization in BCC cells. High levels of Akt expression are seen both in BCC and the adjacent tumor nests (uppermost right). In a BsCC evenly strong nuclear p53 reaction suggests clonal tumor expansion and gene mutation in both components (upper middle right panel; SCC - arrow; BCC - arrowhead). An elevated number of Cyclin D1 (CyclD1) positive cells are detected in actinic keratosis (AktKer; lower middle right panel). Increased caspase-9 expression in an SCC (lowermost right panel) compared to that of the adjacent differentiated epidermis. Immunoperoxidase (brown) reactions. 1st column, x 80; 2nd column, 3rd column, x 100

weakly detected mainly in the undifferentiated basal keratinocytes. Despite obvious labeling of epidermal melanocytes caspase-8 reaction only very weakly labeled all keratinocyte layers. Nuclear p63 expression was the strongest in the basal cells and gradually reduced with keratinocyte differentiation. Nuclear proteins supporting cell replication, including Ki67, cyclin D1 and TopoIIa, as well as the cell cycle regulator p53 protein were found in the basal undifferentiated keratinocytes. Cyclin D3 reaction mostly stained differentiated keratinocytes. When detected, the cyclin dependent kinase (cdk) inhibitor $p21^{waf1}$ was

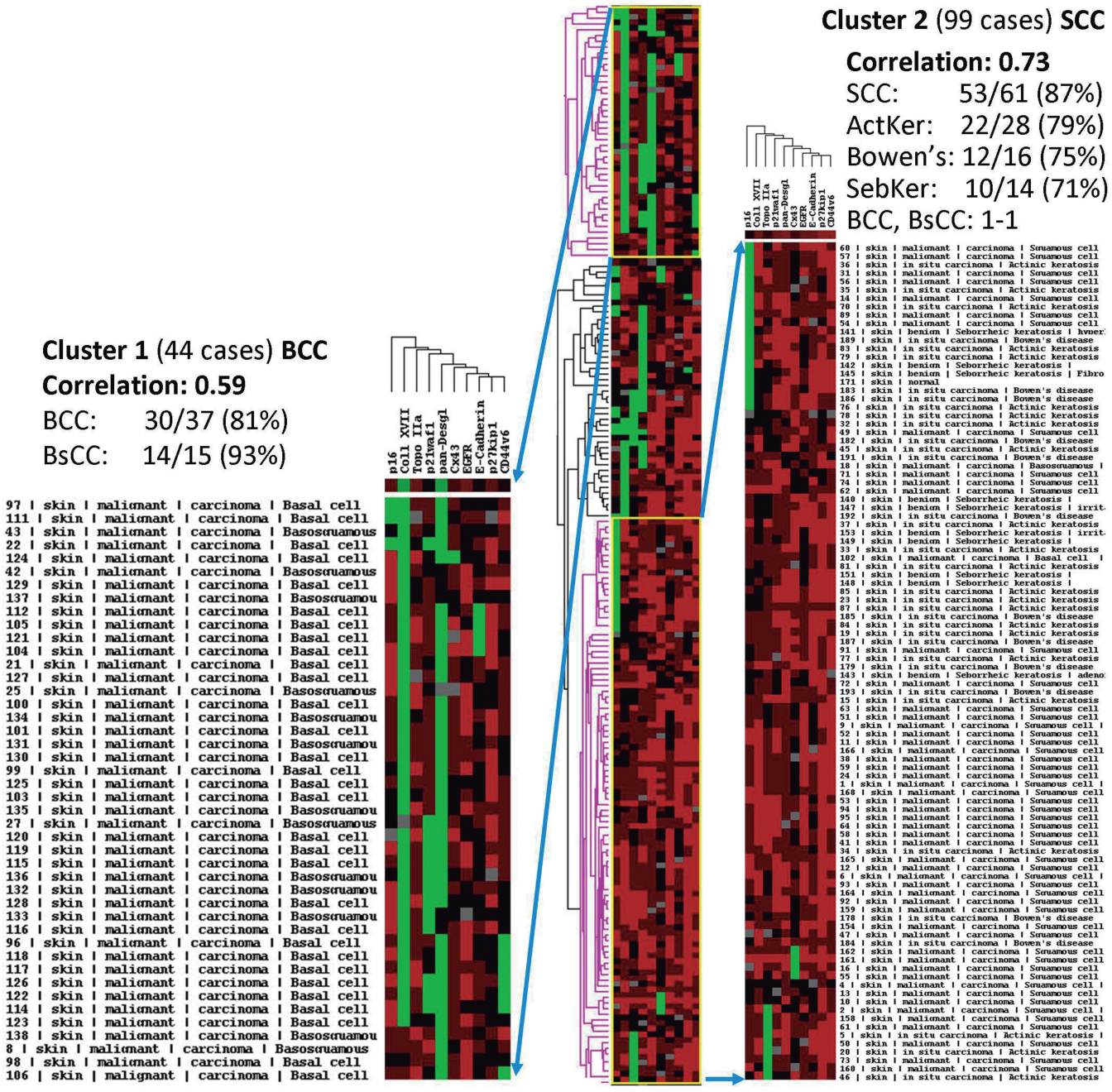

Fig. 4. Hierarchical cluster analysis based on the expression of 10 biomarkers (p16, Collagen XVII, Topoisomerase IIα, $p21^{waf1}$, pan-Desmoglein, connexin43, EGFR, E-Cadherin, $p27^{kip1}$ and CD44v6) that showed statistically differential expression in non-melanoma skin cancer subtypes. The vast majority of basal cell carcinoma (BCC: Cluster 1) and squamous cell carcinoma (SCC: Cluster 2) cases are clustered separately into two major groups.

concentrated in the lowermost suprabasal keratinocytes, while $p27^{kip1}$ reaction showed complementary distribution to that of Ki67 by localizing to the differentiated keratinocytes. The INK family cdk inhibitor $p16^{ink4}$ was hardly found in the normal epidermis except in very few basal keratinocytes.

Biomarker expression in non-melanocytic skin tumors

Table 2 summarizes the mean percent of biomarker positive cases of non-melanocytic skin tumors, including normal skin based on the scoring seen in Table 1. Tested marker profiles of AktKer, Bowen' disease and SCC groups were closer to each other than those of SCC and BCC, while BsCC cases followed the pattern of their dominant component. Malignant tumors displayed similar biomarker expression to undifferentiated basal keratinocytes which differed in many cases significantly from that of differentiated keratinocytes. The cell-cell interaction related membrane proteins were down-regulated, less in SCC than in BCC. Most cell cycle regulatory proteins were expressed at similar levels in BCC and SCC. The expression pattern of biomarkers in SebKer was very similar to that in normal differentiated epidermis except that Ki67, cyclin D1 and TopoIIa positive proliferating cell fractions were more pronounced in SebKer.

Expression of cell interaction related proteins in non-melanocytic skin tumors

Differential protein expression between normal epidermis and skin lesions including SCC and BCC is summarized in Table 2 and demonstrated in Fig. 2. Of proteins involved in keratinocyte-matrix and cell-cell interactions collagen XVII, pan-Desmoglein and CD44v6 were expressed still at relatively high level in SCC but, were hardly found in BCC. Collagen XVII protein was expressed in most samples of AktKer and Bowen's disease. Though cell membrane E-Cadherin was produced at similar levels, it was significantly more delocalized to the cytoplasm in BCC than in SCC. ß-Catenin also showed cytoplasmic delocalization and rare nuclear translocation equally in SCC and BCC. At the same time, Cx43 was expressed at a distinctly lower level in SCC than in BCC, even when both showed high Ki67 positive proliferating cell fractions. Differential expression of Cx43 was obvious in tumor nests with distinct (SCC or BCC) morphology in cases of BsCC.

Expression of cell growth and replication related proteins in non-melanoma skin tumors

Fig. 3 demonstrates typical differential expression patterns of cell growth and replication related proteins in SCC and BCC (see also Table 2). EGFR expression in SCC significantly exceeded that seen in BCC. EGFR, Akt and p53 proteins were upregulated in most SCC and BCC compared to AktKer, Bowen's disease, SebKer and normal keratinocytes. Though DO-7 clone recognizes both the wild type and mutant p53, evenly strong immunostaining in tumor nests potentially indicated the mutant p53 protein. The frequency of p53 positive cases did not differ significantly between well to moderately differentiated (36/48; 75%) and poorly differentiated (9/11; 81%) SCC. Proliferating cell fractions assessed with Ki67, TopoIIa, cyclin D1 and cyclin D3 reactions were also elevated both in SCC and BCC compared to differentiated keratinocytes, SebKer, Bowen's disease or AktKer. The expression of $p21^{waf1}$ was up-regulated mainly in the superficial nests, while $p27^{kip1}$ was evenly down-regulated in skin carcinomas compared to any other groups, and both were expressed at significantly higher level in SCC than in BCC. The expression of cell cycle inhibitor $p16^{ink4}$ protein was also up-regulated both in SCC and BCC but without significant difference. Cytoplasmic delocalization with reduced nuclear expression of $p16^{ink4}$ was frequently seen in skin carcinomas. The expression of pro-caspases caspase-8, caspase-9 was moderately elevated both in SCC and BCC compared to AktKer, Bowen's disease, SebKer and normal epidermis, while the staining of effector caspase-3 was very weak in all tested samples.

Hierarchical cluster analysis of biomarker expression in non-melanoma skin cancer

Cases successfully scored for $2/3^{rd}$ of the markers were tested using unsupervised hierarchical cluster analysis (Fig. 4). Based on the 10 biomarker proteins that showed significantly differential expression (Table 2), clustering highlighted 3 major groups. The largest and most homogeneous cluster incorporated 87% (63/61) of SCC, 79% of AktKer, 75% of Bowen's disease and only 7% (1/15) of BsCC and 3% (1/35) of BCC at relatively high (0.73) correlation. The second cluster included 86% (30/35) of BCC and 93% (14/15) of BsCC, but with a less homogeneous biomarker profile resulting in lower (0.59) correlation coefficient. In the 3^{rd} cluster 6 SCC, 3 AktKer, 1 Bowen's disease and 2 BScC were grouped together with 3 normal basal keratinocyte samples. The rest of the minor additional clusters incorporated 5 AktKer, 4 SebKer, 1 Bowen's disease and 3 BCC with 9 samples of normal differentiated epidermis and 6 normal basal keratinocyte samples with 1 Bowen's disease, respectively. These data showed that BCC and SCC can be clearly separated based on their differential biomarker profiles.

Discussion

In this study, the protein expression profiles of epidermal keratinocyte tumors were tested in TMAs using immunohistochemistry with antibodies specific for proteins involved in cell-matrix and cell-cell interactions, cell cycle regulation and apoptosis. Hierarchical cluster analysis, based on the 9 biomarkers which showed statistically differential expression

between SCC and BCC, clustered SCC, Bowen's disease and AktKer cases exclusively together. This finding supports their common origin and the potential malignant phenotype of AktKer. BCC and BsCC cases were also gathered fully together in line with the dominant basal cell component in BsCC cases tested. These data may reflect the distinct regional and cell type origin of SCC and BCC within the epidermis and the distinct carcinogenic pathways they follow (Tsai and Tsao, 2004; Sellheyer and Krahl, 2008). The fact that practically all BCC were grouped into the same cluster as opposed to SCC, which formed 3 separate clusters (all merged with AktKer and Bowen's disease) also correlates with the more complex genotype associated with SCC compared to BCC development (Boukamp, 2005).

Our comparative *in situ* protein expression data highlight some molecular differences between SCC and BCC in line with their diverse pathobiology, phenotype and clinical behavior (Tsai and Tsao, 2004; Boukamp, 2005). While the abrogation of the ptch-sonic Hedgehog pathway is a genetic hallmark of BCC (Epstein, 2008), diverse oncogenic stimuli and progressive karyotypic complexity characterize SCC development and progression from early *in situ* lesions (Boukamp, 2005; Cassarino et al., 2006). Loss of heterozigosity (LOH) in a number of chromosomal regions is more frequent both in AktKer and SCC than in BCC (Rehman et al., 1996; Waring et al., 1996). As a result, SCC is more dependent on the immune system, since a wider range of keratinocytes with genomic instability are available for transformation into SCC than BCC in immune suppressed patients (Boukamp, 2005).

By linking adjacent keratinocytes and their cytoskeleton tightly, cell adhesion molecules such as desmogleins and E-Cadherin, the Cadherin binding ß-Catenin and the gap junction forming Cx43 contribute both to the architectural and functional integrity of the epidermis (Papadavid et al., 2002; Kurzen et al., 2003; Haass et al., 2006). Down-regulation and delocalization of E-Cadherin and ß-Catenin in line with keratinocyte transformation and SCC progression have been published by several groups (Papadavid et al., 2002; Tanaka et al., 2003; Kudo et al., 2004; Lyakhovitsky et al., 2004; Bosch et al., 2005). Also, deregulated and differential desmoglein isotype expression during SCC progression (Krunic et al., 1998; Kurzen et al., 2003; Brennan and Mahoney, 2009) and marked loss of desmoglein I protein both in BCC and SCC have been described (Tada et al., 2000). However, apart from the selective reduction of CD44 in BCC compared to SCC (Prieto et al., 1995; Karvinen et al., 2003), the differential expression of the rest of cell interaction and growth related proteins we found have not been noted before. These included the significantly reduced levels of collagen XVII, CD44v6, pan-Desmoglein and EGFR and more pronounced cytoplasmic delocalization of EGFR and E-Cadherin in BCC compared to SCC. In line with our findings upregulated EGFR ligand transcripts (amphiregulin, heparin binding EGF and TGF·) and EGFR gene amplification were detected in SCC to offer a target for immunotherapy (Rittie et al., 2007; Uribe and Gonzales, 2011). Since Akt expression did not differ significantly in carcinoma subtypes alternative EGFR activated downstream pathways such as MAPK can also be assumed to support skin carcinoma growth (Greinert, 2009). The significant disruption and cell-cell and cell-matrix interactions and adhesion in BCC is in line with *de novo* direct dermal invasion of BCC as opposed to the gradual progression and invasion from *in situ* lesions of SCC, where cell interaction related proteins remained more preserved (Boukamp, 2005).

A range of connexins including Cx26, Cx30, Cx30.3, Cx31.1, Cx37 and Cx43 have been linked to the regulation of epidermal homeostasis and skin wounding, and connexin mutations were found to result in non-neoplastic skin syndromes (Scott et al., 2012). Furthermore, the up-regulation of Cx26 and Cx30 (Haass et al., 2006) and the down-regulation of Cx43 (Tada and Hashimoto, 1997) have been observed in skin carcinoma. However, the differential expression of Cx43 between BCC and SCC has not been described before. Cx43 is known to integrate keratinocyte functions (Scott et al., 2012) and to control cell growth and tumor invasion (Naus and Laird, 2010). Therefore, the higher expression of Cx43 in BCC than in SCC may contribute to the prevention of metastatic invasion of BCC.

Differential molecular profiles we revealed in BCC and SCC possibly also reflect their supposed distinct regional cell type origin (Sellheyer and Krahl, 2008). Ep-CAM, which is thought to be a marker of follicular differentiation, was exclusively detected in BCC supporting its adnexal (trichoblastic) nature as opposed to the suggested development of SCC from the interfollicular basal keratinocytes (Sellheyer and Krahl, 2008; Ansai et al., 2012).

Accelerated cell proliferation is known to be linked with keratinocyte transformation and skin carcinoma progression (Tilli et al., 2002; Conscience et al., 2006; Utikal et al., 2005). In agreement with this, the expression of cell replication promoting proteins including Ki67, cyclin D1 and TopoIIa we found gradually upregulated in SebKer compared to normal epidermis and through the AktKer, Bowen's disease skin carcinoma progression. However, only TopoIIa positive cell fractions showed statistical difference in skin carcinoma subtypes, they were more frequent in BCC than in SCC. TopoIIa is an essential enzyme of chromosome segregation under the control of wild type p53 (Sandri et al., 1996), thus it is an exploitable target of anticancer therapy (Hande, 1998). In line with earlier studies Ki67 expression also showed a similar trend but without a statistical correlation between our BCC and SCC groups (Tilli et al., 2002; Conscience et al., 2006). It is of note that the low proportion (18%) of poorly differentiated SCC cases involved in our cohort showed

at least as high proliferating fractions as the more homogenous nodular BCC group. Cyclin D3, which can reduce cyclin D2 and protect keratinocytes from Ras-dependent carcinogenesis (Rojas et al., 2007), was detected more in differentiated than basal keratinocytes and its expression in SCC and BCC did not differ significantly.

Skin cancer development is also connected with the abnormal expression of genes involved in the control of cell cycle and cell fate (Abd Elmageed et al., 2009). The master regulator of cell cycle control, DNA damage repair and apoptosis p53 is known to be frequently upregulated and/or mutated, both in SCC and its precursors and BCC, but even in the normal sun-exposed skin (Backvall et al., 2005; Abd Elmageed et al., 2009). Therefore, p53 mutation alone may not be enough to drive skin carcinogenesis. Also, it can not be traced with immunohistochemistry since available antibodies cannot differentiate between the upregulated normal or non-functioning mutant protein (Mineta et al., 1998), and because some truncated p53 mutant cases show false negativity (Eicheler et al., 2002). In our cohort p53 protein was over-expressed in skin carcinomas compared to normal epidermis and SebKer but the frequency of p53 positive cells did not differ significantly in the skin carcinoma subtypes, suggesting a similar contribution to SCC and BCC development. In agreement with the crucial role of p63 in the maintenance of basal cell population in stratified epithelia (Reis-Filho et al., 2002) its expression inversely correlated with normal keratinocyte differentiation and showed equally high levels in AktKer, SCC and BCC.

Of the cyclin dependent kinase (cdk) inhibitors $p16^{ink4}$ and $p21^{waf1}$ are downstream targets of wild type p53 activation which can be coordinately induced in sun (UV) exposed skin (Abd Elmageed et al., 2009). LOH affecting the CDKN2A gene that encodes $p16^{ink4}$ protein to block cyclin D1/CDK4/6 complex and cell proliferation is frequent in skin carcinoma, including *in situ* disease (Boukamp, 2005). Despite this, the vast majority of studies, including ours, found elevated $p16^{ink4}$ expressing cell fractions in AktKer, Bowen's disease, SCC and also in BCC, which was useful to differentiate these from benign lesions such as SebKer (Salama et al., 2003; Conscience et al., 2006). $p16^{ink4}$ expression was highly heterogeneous, mostly concentrated in the invasive tumor edges both in SCC and BCC with frequent cytoplasmic delocalization. The nuclear reaction has been suggested to accompany SCC progression and invasiveness (Hodges and Smoller, 2002; Nilsson et al., 2004). Delocalized $p16^{ink4}$ protein, however, may not be functional, or may serve unknown cell cycle control independent functions.

$p21^{waf1}$, which normally blocks retinoblastoma phosphorylation upon p53 induction and arrests the cell cycle at G1 phase, was expressed independently of p53 (Tron et al., 1996). This was in agreement with the supposed dual role of $p21^{waf1}$, since it has also been implicated in blocking terminal keratinocyte differentiation and thus promoting skin carcinogenesis (Di Cunto et al., 1998). Similar to other groups we detected high $p21^{waf1}$ positive cell fractions in squamo-proliferative lesions (Wrone-Smith et al., 1999; Tron et al., 1996). The fact that $p21^{waf1}$ expression was more frequent in the superficial than vertically spreading tumor nests suggests its role either in cell cycle inhibition or early carcinogenesis rather than in tumor invasion. As opposed to this the other cdk inhibitor of the same family, $p27^{kip1}$, was evenly down-regulated in proportion with carcinoma dedifferentiation. Since neither $p21^{waf1}$ nor $p27^{kip1}$ genes are known to be mutated, their lower protein levels in BCC than in SCC can be linked either to the less differentiated phenotype of the tested BCC cases and/or their differential contribution to BCC compared to SCC development.

Deregulated programmed cell death response has also been associated with skin carcinoma development (Erb et al., 2005). The extrinsic apoptosis pathway is mediated through the activation of pro-caspases caspase-8 and caspase-9 and the effector caspase-3. Caspase-8 and caspase-9 we found moderately but equally upregulated both in SCC and BCC compared to differentiated epidermal keratinocytes, which might argue against their supposed inhibition of programmed cell death response in skin cancer. However, the loss of caspase-8 was shown to induce wound healing response (Lee et al., 2009) and inflammatory skin disease (Kovalenko et al., 2009) implying that caspase-8 expression can shelter keratinocytes from the immune response. Furthermore, Fas-L upregulation that can normally mediate extrinsic apoptotic signaling through Fas (receptor) and the downstream caspase cascade was also revealed to contribute to the immune escape in invasive skin carcinoma (Bachmann et al., 2001). Therefore, the elevated caspase levels we detected in skin carcinoma are more likely to support tumor protection from the immune response than apoptosis induction. In addition, the intrinsic programmed cell death response mediated by proteins of the bcl-family was also found to be inhibited in skin carcinoma by elevated bcl-2 and bcl-x expression (Wrone-Smith et al., 1999).

Inconsistent *in situ* protein expression data gained in independent studies usually result from using diverse patient cohorts, antibody clones, antigen retrieval techniques and detection methods. TMA studies combined with digital microscopy allow highly efficient expression profiling of a series biomarkers in many tissue samples at standard conditions for reliable results (Kononen et al., 1998; Krenacs et al., 2010).

In conclusion, differential protein expression profiles of epidermal keratinocyte tumors were revealed in correlation with their distinct pathobiology and histomorphology. Particularly notable is the more pronounced down-regulation of cell adhesion molecules in BCC that may contribute to its *de novo* dermal

invasion compared to the gradual dermal invasion of SCC with more preserved cell adhesion machinery. The abnormal but differential expression of some cell cycle regulatory molecules in skin carcinoma subtypes also suggests that deregulation of cell replication also follows fairly diverse pathways in SCC and BCC. Detection of differentially expressed proteins in skin carcinoma can be utilized as surrogate markers for diagnosing borderline tumors.

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