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Report

Differential expression patterns of metastasis suppressor proteins in basal cell carcinoma

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Abstract

Background Basal cell carcinomas (BCCs) are common malignant skin tumors. Despite having a significant invasion capacity, they metastasize only rarely. Our aim in this study was to detect the expression patterns of the NM23-H1, NDRG1, E-cadherin, RHOGDI2, CD82/KAI1, MKK4, and AKAP12 metastasis suppressor proteins in BCCs.

Methods A total of 96 BCC and 10 normal skin samples were included for the immunohistochemical study. Eleven frozen BCC samples were also studied by quantitative real time polymerase chain reaction (qRT-PCR) to detect the gene expression profile.

Results NM23-H1 was strongly and diffusely expressed in all types of BCC. Significant cytoplasmic expression of NDRG1 and E-cadherin was also detected. However, AKAP12 and CD82/KAI1 expression was significantly decreased. The expressions of the other proteins were somewhere between the two extremes. Similarly, qRT-PCR analysis showed down-regulation of *AKAP12* and up-regulation of *NM23-H1* and *NDRG1* in BCC. Morphologically aggressive BCCs showed significantly higher cytoplasmic NDRG1 expression scores and lower CD82/KAI1 scores than non-aggressive BCCs.

Conclusion The relatively preserved levels of *NM23-H1*, NDRG1, and E-cadherin proteins may have a positive effect on the non-metastasizing features of these tumors.

Introduction

Basal cell carcinoma of the skin (BCC), a common human carcinoma, tends to be locally invasive and metastasizes only rarely.[1] Despite the low metastasizing ability, these lesions show significant invasion capacity if neglected.[2] This unusual predisposition makes BCC an interesting biological model for invasion and metastasis.

Metastasis is a complex biological process and is controlled by various mechanisms. One important mechanism is provided by metastasis suppressor proteins (MSPs). MSPs inhibit or suppress metastasis without any effect on cell proliferation and affect different steps of the complex metastasizing process. To date, more than 30 MSPs have been identified.[3] In this study, we investigated the expression patterns of seven well-defined important MSPs, including NM23-H1, NDRG1, E-cadherin, RHOGDI2, CD82/KAI1, MKK4, and AKAP12 in BCCs.

NM23-H1 is the first described MSP and is downregulated in several metastatic cell lines and in a group of human carcinomas.[4] *NM23-H1* gene encodes a nucleoside diphosphate kinase A. Although the metastasis suppressor mechanism of NM23-H1 is not clear, its interaction with kinase suppressors of RAS and, as a result, alteration of the MAPK signaling pathway is a probable mechanism.[3] It has also recently been suggested that it suppresses metastasis by inhibiting the expression of EDG2 (lysophosphatidic acid receptor).[5]

NDRG1 (N-myc downstream regulated 1) is a member of the NDRG family of proteins and has been shown to reduce metastasis in colon, breast, and prostate neoplasms.[6] Although the metastasis suppressor mechanism of NDRG1 is not clear, the interaction with the cell–cell adhesion molecules β-catenin and E-cadherin might be a possible mechanism.[7,8]

E-cadherin is a well-known cell–cell adhesion protein, and loss of its expression plays important roles in tumor

invasion and metastasis.[9,10] It also functions as a negative regulator of the canonical WNT signaling pathway. E-cadherin has been extensively studied in human tumors, including BCCs.[11,12]

RHOGDIs (Rho GDP-dissociation inhibitors) make up a small group of proteins that negatively control RhoGTPases, which play important roles in cancer and metastasis.[13] RHOGDI2 acts as an MSP in bladder tumors and probably in other types of epithelial tumors.[14] However, RHOGDI2 may have cancer or tissue-specific functions in tumor suppression, while it might promote cancer invasiveness in a minority of human cancers. [14,15]

The CD82/KAI1 protein, also called TSPAN27, is a member of the tetra-spantin family, which has important roles in adhesion, motility, and tumor progression.[16,17] It was initially demonstrated as an MSP in prostate carcinoma.[18] The prognostic importance of this protein was then demonstrated in other human cancer types.[17,19,20]

Mitogen-activated protein kinase kinase 4 (MKK4) is a component of MAP kinase in stress-activated protein kinase signaling.[21,22] It was identified as an MSP in prostate and ovarian cancer.[23,24] Although the tumor or metastasis suppressor function of MKK4 is generally accepted, there are also some clues that it has pro-oncogenic roles.[22]

AKAP12, also called SSeCKS/Gravin, is a scaffold protein and functions as a binding partner of protein kinase C and A, calmodulin, F-actin, cyclins, Src, and phospholipids.[25] Significant clinical and experimental evidence has shown that AKAP12 is an important tumor and metastasis suppressor.[25] AKAP12 expression is downregulated in various solid human cancers and some leukemias.[25,26]

The significance of MSPs in BCC is not well known due to the limited number of studies (Table 1). Our aim was to demonstrate the distribution and expression of the seven important MSPs in BCC. We also tried to determine the relationship between protein expression levels, p53 status, and well-known clinicopathological parameters.

Materials and methods

Study group

A total of 96 BCCs from 92 patients (47 male/45 female) were included in this study. All patients were Caucasian, and the mean age was 66.3 ± 13.4 years. All lesions were excised from the head and neck area except for five lesions from the trunk. Normal epidermis samples adjacent to the BCCs (NE-BCC) and 10 non-lesional, histopathologically confirmed normal skin tissues (N) were also studied. As there is no easy way to subclassify BCCs because of their wide and heterogeneous morphological spectrum, we used the criteria summarized by Carr *et al.* for classifying our group.[27] Two major tumor groups were created and immunohistochemically scored to establish the differential expression patterns and contribution of the proteins:

1 Morphologically non-aggressive BCC types, including nodular, adenoid, superficial, and mixed carcinomas with less than 50% infiltrative pattern (n = 68).
2 Morphologically aggressive BCC types, including infiltrative BCCs with/without desmoplasia and mixed carcinomas with more than 50% infiltrative pattern (n = 28).

Clinicopathological features

The conventional clinicopathological parameters, including maximum diameter of the tumor, invasion depth, perineural invasion, anatomical invasion (Clark's) level, and local recurrences, were investigated. Tumor-associated inflammation was graded as previously described by Kaur *et al.*[28]

Immunohistochemistry

The classical, labeled streptavidin–biotin immunohistochemistry technique (UltraVision/DAB-Thermo Scientific, Waltham, MA, USA) was used for immunostaining of the slides. All steps of immunostaining were carried out by specific capillary cover-plate technology in a Thermo-Shandon Sequenza® (Waltham, MA, USA) manual staining device. The negative control was performed by skipping the primary antibody step. Vendors, incubation time, antigen retrieval solutions, and positive controls are demonstrated in Table 2.

Immunohistochemical analyses

The immunohistochemistry results were analyzed semiquantitatively by using an immunohistochemical histological score (HSCORE) that included both the intensity and proportional distribution of specific staining. Based on a specific method described by McCarty *et al.*, the HSCORE has been formulated as $HS = \sum (P_i \times i/100)$, where P_i indicates the percentage of stained cells (0–100%) at each intensity and i shows the intensity of staining and ranges from no staining (0 points) to strong staining (3 points).[29] The calculated HSCOREs were between 0 (no staining) and 300 (strong-diffuse staining) points. All calculations were performed with the Microsoft Office Excel® program using a simple macro. After evaluating the whole slide for specific staining, a minimum of five to seven randomly selected areas at medium power magnification (×20) in normal and neoplastic tissues were analyzed for HSCORE. Nuclear ($_{nuc}$) and cytoplasmic ($_{cyt}$) expressions were evaluated separately for NM23-H1, MKK4, RHOGDI2, and NDRG1. Only membranocytoplasmic staining of E-cadherin, AKAP12, and CD82, and nuclear staining of p53 were accepted as positive.

Statistical analysis

Statistical analyses were performed using the PASW® Statistics 18 software (Chicago, IL, USA). The "Bonferroni correction" was applied for reducing the false-positive results. The differences between the HSCOREs of the groups were studied

Table 1 Literature summary of MSPs in BCCs which were studied in this article

Gene/protein	Author/year/publication	Case distribution	Results	Comment/other
NM23-H1	Ro YS, Jeong SJ. *J Korean Med Sci* 1995; **10**: 97–102	25 BCC, 26 SCC, 9 KA	All of the BCCs positive with different proportions.	Positivity of NM23 were more intense in SCC and KA than BCCs.
NM23-H1	Kanitakis J, *et al. J Cutan Pathol* 1997; **24**: 151–156	28 BCC, total 104 benign and malignant skin lesion	All of the BCCs showed significant positivity.	SCCs showed weak positivity.
E-cadherin	Pizarro A, *et al. Br J Cancer* 1994; **69**: 157–162	31 BCCs (8 NBCC, 8 SBCC, 15 IBCC)	All of the BCCs positive, NBCC and SBCCs showed preserved levels but IBCC showed reduced levels.	Statistically significant correlation between reduction in E-cadherin expression and the infiltrative growth pattern.
E-cadherin	Pizarro A, *et al. Br J Cancer* 1995; **72**: 327–332	32 BCCs (14 NBCC, 7 SBCC, 11 IBCC)	Infiltrative BCCs showed reduced E-cadherin levels. NBCC stained more heterogeneously. SBCC showed generally preserved levels.	P-cadherin staining was seen significantly protected in all types of BCCs.
E-cadherin	Fuller LC, *et al. Br J Dermatol* 1996; **134**: 28–32	30 BCC,16 SCC, 6 BD, 10 other skin lesions	28 of 30 BCC showed reduced expression.	Expression also reduced in SCCs but not BD and other skin lesions.
E-cadherin	Tada H, *et al. J Dermatol* 1996; **23**: 104–110	11 BCCs (8 NBCC, 2 IBCC,1 SBCC), 7 SCC, 8 PD, 2 BD, 3 TC	All of the BCCs positive. Expression strong as that of normal epidermis.	SCC, BD showed no positivity. PD expressed weak positivity. TC showed positivity.
E-cadherin	Shirahama S, *et al. J Dermatol Sci* 1996; **13**: 30–36	10 BCC, 9 SCC, 6 MM,5 PD	All of the BCCs showed preserved expression. No infiltrative BCC included.	SCCs showed reduced expression, MM and PD showed no positivity. There was no difference in soluble E-cadherin expression in BCC type.
E-cadherin	Kooy AJ, *et al. Hum Pathol* 1999; **30**: 1328–1335	15 BCC (9 NBCC, 2 nodular/ adenoid BCC, 2 nodular superficial BCC, and 2 SBCC)	More than 70% of the BCC cells showed expression in all BCCs. The intensity of E-cadherin in BCC compared with epidermis was not statistically significant.	A significantly reduced expression of α-catenin and CD44V6 in BCCs
E-cadherin	Koseki S, *et al. J Dermatol* 2000; **27**: 307–311	25 BCCs, 11 SCCs, 9 KA, and 11 BD	E-cadherin expression is preserved in BCC.	AMeX (acetone-methylbenzoate-xylene) method was used. Expression also preserved in BD but dowregulated in SCC and KA.
E-cadherin	Fukumaru K, *et al. J Dermatol* 2007; **34**: 746–753	86 BCC	Moderate or strong expression detected in all of the BCCs.	Nuclear β-catenin was identified in 20 of 86 cases
E-cadherin	Uzquiano MC, *et al. Mod Pathol* 2008; **21**: 540–543	12 NBCC, 10 IBCC, and 10 metastatic BCC	Present in 75% of the NBCC, 70% of IBCC, and all of the metastatic BCC. ($P < 0.05$ for metastatic vs. nodular.)	Actin and calponin also studied. Increased actin may contribute to local invasiveness, but it is lost in the metastatic phenotype.
E-cadherin	Papanikolaou S, *et al. Histopathology* 2010; **56**: 799–809	100 BCC	E-cadherin was found in 71% of cases while nuclear immunoreactivity was also observed in 90%.	Snail, nuclear β-catenin and α-SMA were detected in 100, 99, and 97% of BCCs, respectively. Aberrant expression of E-cadherin, nuclear β-catenin and α-SMA correlated with BCC tumor invasion.
E-cadherin	Brinkhuizen T, *et al. PLoS One* 2012; **7**: e51710	59 BCC	Lowered expression than normal epidermis were seen in all of the BCCs. Intensity of staining was rated as strong (69.5%) and staining was independent of BCC subtype.	Absence of nuclear β-catenin in many cases may be due to high E-cadherin levels.

Table 1 (Continued)

Gene/protein	Author/year/publication	Case distribution	Results	Comment/other
E-cadherin	Tucci MG, *et al. Arch Dermatol Res* 2013 epub	30 BCC (10 SBCC, 9 NBCC,11 IBCC)	Its expression in BCCs was lower than in normal skin. E-cadherin staining was significantly reduced in infiltrative BCC.	Cdc42 protein were also studied and showed upregulation in BCCs.
CD82	Okochi H *et al. Br J Dermatol* 1997; **137**: 856–863	5 BCC 5 SK, 3 BD	CD82 was markedly downregulated or completely negative in BCCs.	BD showed similar strength as normal. SK showed positivity but downregulated. Other tetraspantins CD9 and CD81 were also downregulated in BCCs.
AKAP12	Wu W, *et al. Clin Exp Dermatol* 2011; **36**: 381–385	85 BCC,67 SCC,43 AK	The methylation frequencies of AKAP12 were significantly higher than those of normal tissues.	No immunostain or Western blot only methylation study.

AK, actinic keratosis; BCC, basal cell carcinoma; BD, Bowen disease; IBCC, infiltrative BCC; KA, keratoacanthoma; MM, malignant melanoma; NBCC, nodular BCC; PD, Paget disease; SBCC, superficial BCC; SCC, squamous cell carcinoma; SK, seborrheic keratosis; TC, trichilemmal carcinoma.

with the non-parametric Mann–Whitney *U* test. The correlation between the parameters was investigated by Spearman's correlation test, and $r \geq 0.25$ and $P \leq 0.05$ were accepted as a significant correlation.

Quantitative real-time polymerase chain reaction study group

Quantitative real-time polymerase chain reaction (qRT-PCR) experiments were performed for frozen tissue consisting of 11 BCCs, three normal non-lesional skins, and eight normal skins adjacent to the BCCs. All tissues were re-confirmed by frozen sections before RNA isolation.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from tissues using commercial RNA extraction kit (Fibrous Tissue kit; Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. A total of 500 ng of total RNA was reverse-transcribed using oligo-dT primers for cDNA synthesis. qRT-PCR experiments were performed using the SYBR® Green chemistry in 96-well reaction plates with optical caps (Bioplastics, Landgraaf, Netherland) in an MX3005P (Strategene®-Agilent, Santa Clara, CA, USA) thermocycler. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *HPRT1* (hypoxanthine phosphoribosyltransferase 1) genes were selected as reference genes. The qRT-PCR reaction contained 10 μl 2 $\times$SYBR Green PCR Master Mix (Finnzyme-Thermo, Waltham, MA, USA), forward and reverse primers at optimized concentrations of 300 nM (150 nM for *NDRG1* primers, 200 nM for *MKK4* primers), 2 μl cDNA template (500 ng/ml), and PCR grade water up to a final volume of 20 μl. The fluorescence data were also confirmed by melt-curve analysis for the specificity of the product. The primers used in this study are documented in Table 3.

Quantitative real-time polymerase chain reaction data analysis

Data analysis was performed with the free-use REST© 2009 (Qiagen) software, which gives reliable results in small study groups.[30] This software uses the classic formulation

$$\text{Ratio} = (E_{\text{target}})^{\Delta CP}{}_{\text{target (control - sample)}} / E_{\text{ref}})^{\Delta CP}{}_{\text{ref (control - sample)}}$$

where *E* is the amplification efficiency of the primers and CP is the cycle threshold, and shows both fold changes and standard errors between the controls and the samples. Statistical significance between the groups was evaluated by the Pair Wise Fixed Reallocation Randomization Test© using the REST software.

Results

Immunohistochemical staining

In normal epidermis (N), all of the proteins were expressed at various intensities. NDRG1, E-cadherin, RHOGDI2, and cytoplasmic NM23-H1 positivity was

Table 2 Primer antibodies used in this study

Antibody	Vendor	Dilution	Antigen retrieval	Incubation	Control tissue
RHOGDI2	Abcam	1/100	Citrat; pH 6	Overnight	Tonsil
NM23-H1	Abcam	1/200	No	Overnight	Ductal carcinoma, breast
MKK4	Novocastra; Leica	1/20	Citrat; pH 6	Overnight	Ductal carcinoma, breast
CD82	Novocastra; Leica	1/20	Citrat; pH 6	Overnight	Tonsil
AKAP12	Atlas	1/100	Citrat; pH 6	Overnight	Testis
NDRG1	Santa Cruz	1/100	EDTA; pH 9	Overnight	Placenta
E-cadherin	Cell Signaling	1/100	Citrat; pH 6	Overnight	Adenocarcinoma, colon
P53	Thermo	1/100	Citrat; pH 6	Overnight	Adenocarcinoma, colon

Table 3 qRT-PCR primer sequences used in this study

Gene	F	R
GAPDH	5′-AGGTGAAGGTCGGAGTCAAC-3′[a]	5′-GGGTCATTGATGGCAACA-3′
HPRT1[b]	5′-GCTGACCTGCTGGATTACAT-3′	5′-CCCTGTTGACTGGTCATTAC-3′
KAI1/CD82	5′-AGCAGAACCCGCAGAGTCCT-3′	5′-CTTCCACGAAACCAGTGCAG-3′
MAP2K4[c]	5′-AGTGGACAGCTTGTGGACTCT-3	5′-AACTCCAGACATCAGAGCGGA-3′
NM23 (NME1)	5′-CCTGAAGGACCGTCCATTCT-3′	5′-CCGTCTTCACCACATTCAGC-3′
E-cadherin (CDH1)	5′-GTCCTGGGCAGAGTGAATTT-3′	5′-TCTGTGCCCACTTTGAATCG-3′
AKAP12	5′-TCACAGAGGTTGGACAGAGA-3′	5′-GTGAACAACCGCTGACTTAG-3′
RHOGDI2 (ARHGDIB)	5′-CCTCCACCACAGAAGTCCCT-3′	5′-GCTTTCGGATCTGTCACCAC-3′
NDRG1	5′-CAAGATCTCAGGATGGACC-3′	5′-GACCACTTCCACGTTACTC-3′

[a]Mol Cancer 2010; 9: 226.
[b]Designed by Dedeoğlu BG, PHD.
[c]*Gynecol Oncol* 2007; 105: 312–320.

strong and easily detectable. However, nuclear NM23-H1 was only seen in the basal layers of the epidermis. AKAP12, CD82/KAI, and cytoplasmic MKK4 were stained at medium intensities. Nuclear staining of MKK4 was very weak and not easily detectable. NE-BCC showed more heterogeneous positivity with all antibodies when compared to the normal epidermis.

In BCCs, both cytoplasmic ($NDRG1_{cyt}$) and nuclear NDRG1 ($NDRG1_{nuc}$) positivity were homogeneous (Fig. 2c, d). $NDRG1_{cyt}$ was seen in all BCCs. However, only 74 of 96 (77%) BCCs showed nuclear positivity. Similarly, NM23-H1 cytoplasmic expression (NM23-H1cyt) was also strong and diffused, except in two BCCs (97.9%) (Fig. 2a, b). Nuclear expression of NM23-H1 was weaker and expressed in 73 of 96 (76%) cases. E-Cadherin antibody was represented by both membranous and cytoplasmic positivity except in seven BCCs (92.7%) (Fig. 2e, f). Nuclear staining was seen very rarely and usually in strongly stained areas. CD82/KAI and AKAP12 positivity was significantly reduced and only seen in focal areas of the BCCs in 14 (15.1%) and 21 of 96 (21.8%) cases, respectively (Fig. 3c–f). MKK4 immunostaining of neoplastic tissues was weak/medium cytoplasmic positive in 73 (76%) and weak nuclear positive in only 35 (36.4%) cases (Fig. 3a,b). The nuclear expression was more evident in normal tissues. Although RHOGDI2 staining showed cytoplasmic positivity ($RHOGDI2_{cyt}$) in 89 of 96 (92.7%) cases, the intensity was significantly reduced (Fig. 2g,h). Nuclear expression ($RHOGDI2_{nuc}$) was very weak and heterogeneously in 59 (61.4%) of 96 BCCs.

HSCORES

The HCORES of the groups are demonstrated in the boxplot graph (Fig. 1).

Statistical results

As the tumor microenvironment changes are part of carcinogenesis, we evaluated the difference between the NE-BCC and normal non-lesional (N) skin. Although there was no statistical difference for $RHOGDI2_{cyt}$, $NM23\text{-}H1_{cyt/nuc}$, CD82, and MKK4 $_{cyt/nuc}$ scores between the two groups, the other markers showed a significant reduction in NE-BCC ($P \leq 0.05$).

When normal epidermis was compared to BCCs, all of the scores except for $NM23_{cyt/nuc}$ were significantly lower in the tumor groups ($P \leq 0.01$). Similarly, when NE-BCC was compared to BCCs, all of the markers except for $NM23\text{-}H1_{cyt}$ and $NDRG1_{cyt}$ indicated significantly reduced scores ($P \leq 0.01$) in the BCC group. Furthermore, $NM23\text{-}H1_{nuc}$ scores were higher in BCCs than in NE-BCC.

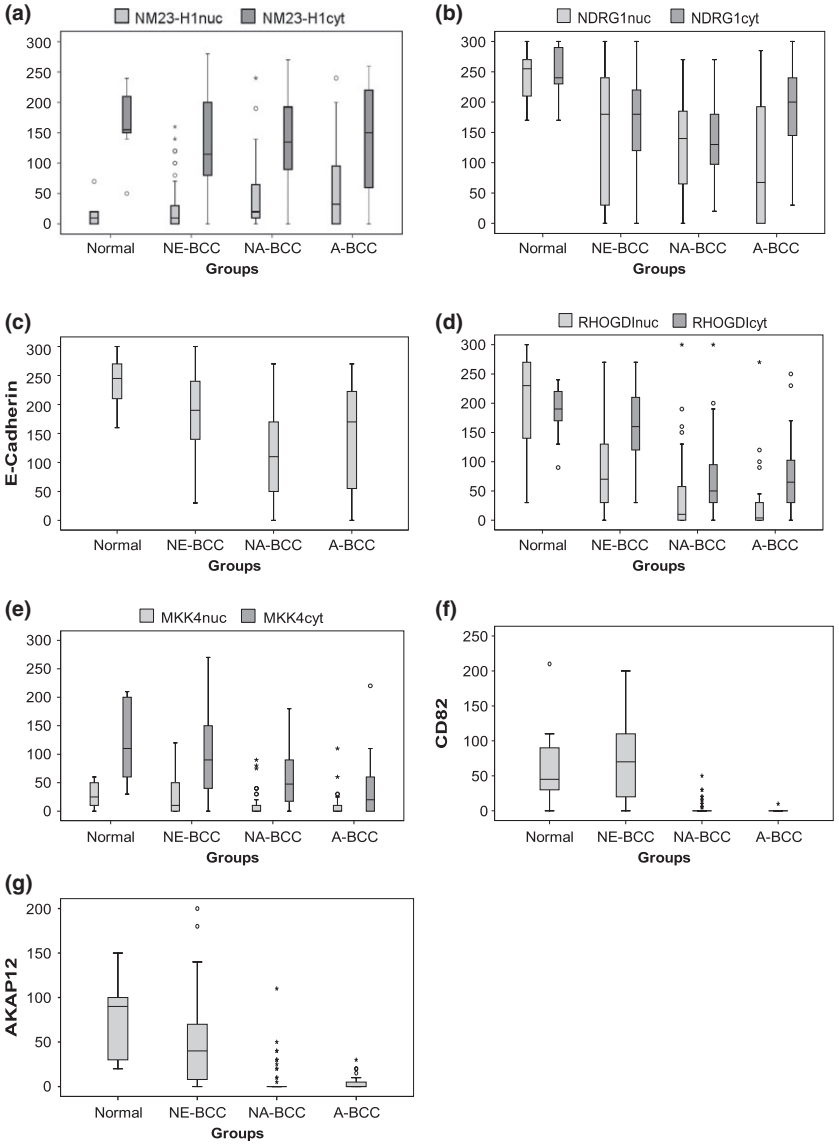

Figure 1 Boxplot graphics of the groups. (a) NM23-H1; (b) NDRG1; (c) E-cadherin; (d) RHOGDI2; (e) MKK4; (f) CD82; (g) AKAP12. The protected levels of NM23-H1 (a), NDRG1 (b) and E-cadherin (c) in BCCs are clearly demonstrated in boxplot graphics. Conversely, significant downregulation or lost of CD82 (f) and AKAP12 (g) HSCOREs have attracted attention in the tumor group. For RHOGDI2 (d) and MKK4 (e) HSCOREs, although the downregulation is seen in both nuclear and cytoplasmic scores, reduction of nuclear HSCOREs are more significant. A-BCC, aggressive BCC; BCC, basal cell carcinoma; NA-BCC, non-aggressive BCC; NE-BCC, normal epidermis adjacent to BCC. * and ° demonstrate more than one case with similar HSCORE

Morphologically aggressive BCCs expressed significantly higher $NDRG1_{cyt}$ scores ($P = 0.001$) and lower CD82/KAI1 scores ($P = 0.048$).

BCCs with perineural invasion showed lower nuclear NM23-H1 levels ($P = 0.01$). Recurrent BCCs expressed higher $RHOGDI2_{nuc}$ levels ($P = 0.01$) when compared to the non-recurrence group.

Correlation analysis

In the BCC group, there were significant correlations ($P = 0.01$ level) between several markers as follows: $NM23\text{-}H1_{nuc}$-$NM23\text{-}H1_{cyt}$ ($r = 0.442$); $AKAP12$-$RHOGDI2_{cyt}$ ($r = 0.333$); $AKAP12$-$NDRG1_{cyt}$ ($r = 0.280$); E-cadherin-$RHOGDI2_{cyt}$ ($r = 0.303$); E-cadherin-$NDRG1_{cyt}$ ($r = 0.413$); $RHOGDI2_{nuc}$-$RHOGDI2_{cyt}$ ($r = 0.405$); $RHOGDI2_{nuc}$-MKK4cyt ($r = -0.294$); $NDRG1_{nuc}$-$NDRG1_{cyt}$ ($r = 0.356$); and $MKK4_{nuc}$-$MKK4_{cyt}$ ($r = 0.365$). There were also significant negative correlations between AKAP12 and inflammation ($r = -0.275$; $P = 0.007$).

A randomly selected subgroup of 44 BCCs from the main group was stained with p53 primary antibody to establish the correlation between MSPs and p53. We detected only a negative correlation with $RHOGDI2_{cyt}$ ($r = -0.316$; $P = 0.037$).

Relative expression software tool (REST) analysis of quantitative real-time polymerase chain reaction data

BCCs were compared to both the normal non-lesional skin tissue and the skin adjacent to neoplasia groups. We found significant upregulation of *NM23* (1.4-fold, $P = 0.032$) and downregulation of *AKAP12* (−1.2-fold; $P = 0.006$) when BCC was compared to normal skin.

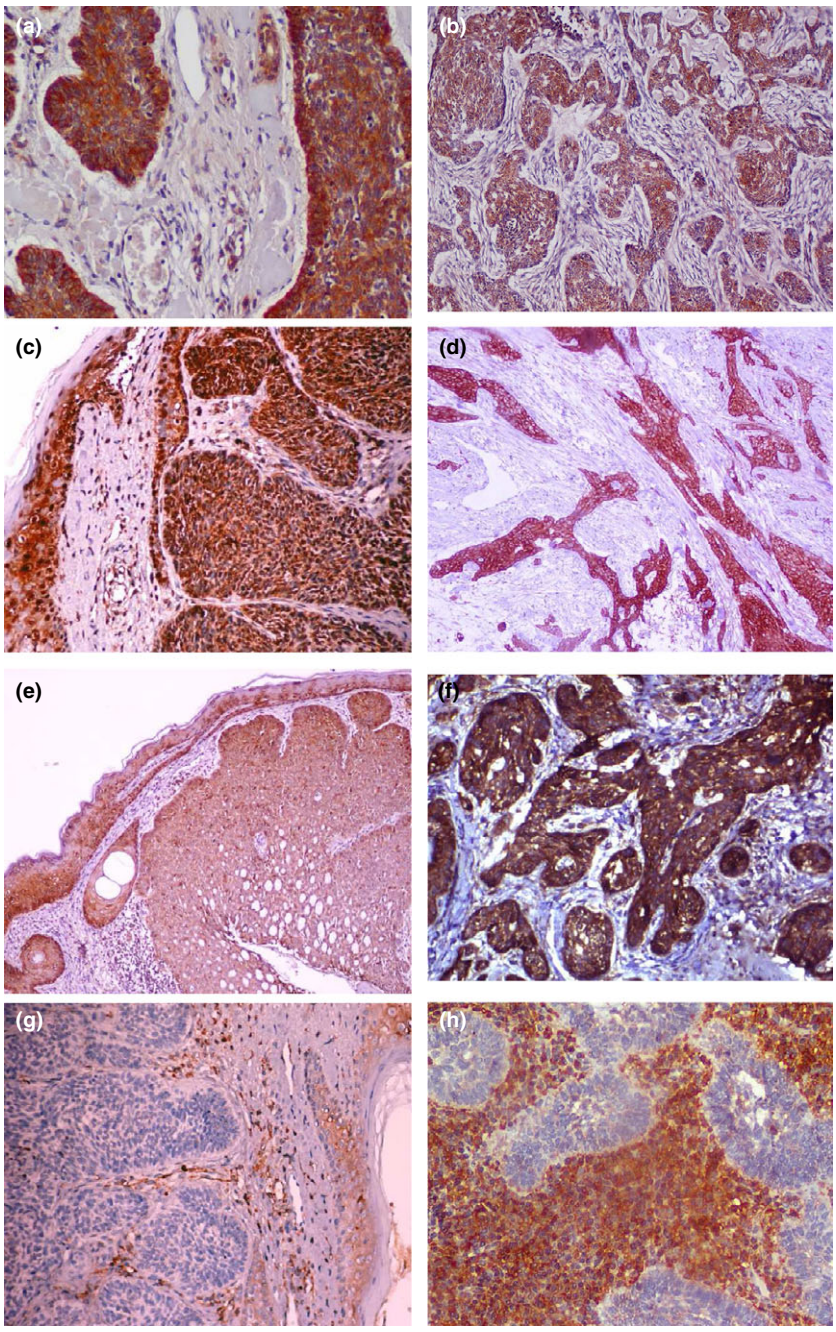

Figure 2 Significant NM23-H1 positivity is seen in both nodular (a) and infiltrative basal cell carcinomas (BCCs). (b). NM23-H1 immunostaining highlights basal palisading cells in nodular type BCC. (c,d) Similar to NM23, strong NDRG1 expression is detected in BCCs. (e,f) E-cadherin expression is protected in BCCs. (g,h) RhoGDI expression is very weak or negative in BCCs. The contrast between positive inflammatory cells and carcinoma cells is clearly demonstrated (h). (a,f, h, ×200; b–e,g, ×100)

NDRG1 showed statistically significantly higher levels (2.2-fold, $P = 0.001$) in BCC when compared to the skin adjacent to the neoplasia, similar to the immunohistochemical results.

Discussion

Invasion and metastasis are the most important hallmarks of cancer and well-known clinical signs of a poor prognosis.[31,32] BCCs display all the hallmarks of cancer, including invasion, with the exception of metastasis. To date, the question of why BCCs metastasize only rarely has not been adequately answered. A possible but unsupported explanation for this question is the strict stromal dependence of BCC.[33] In this study, we examined the contribution of the MSPs in the non-metastasizing feature of BCCs and focused on seven well-known proteins.

We detected that NM23-H1 HSCOREs were protected in BCCs, and mRNA level of *NM23-H1* was higher in BCCs than in normal skin. Similarly, Ro and Jeong and

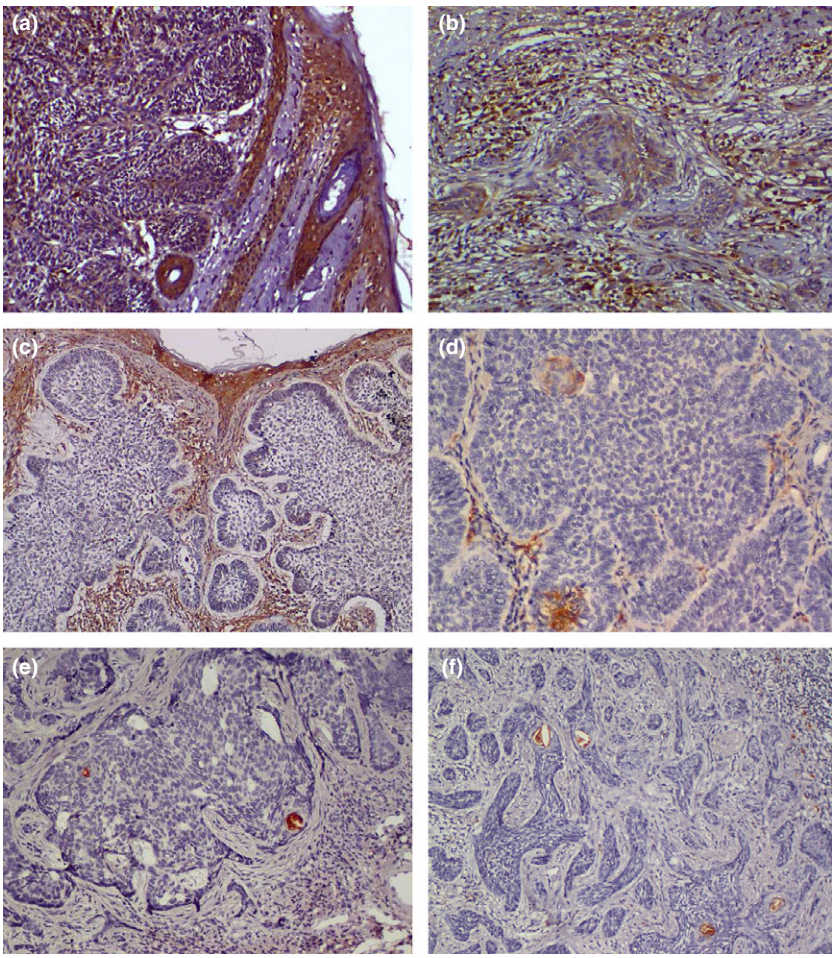

Figure 3 Normal skin adjacent to basal cell carcinoma (BCC) expresses medium strength nuclear and cytoplasmic MKK4 staining. Nodular (a) and infiltrative BCCs (b) show reduced nuclear and cytoplasmic staining. (c) AKAP12 expression is clearly seen in normal epidermis and stromal cells but not in BCCs. (d) Weak AKAP12 staining is detected only at squamous differentiation areas in a BCC case. Stromal cells are also positive in this case. CD82 expression is not detected in nodular (e) and infiltrative (f) BCCs except in focal squamous differentiation areas. (a,c,f, ×100; b,d,e, ×200)

Kanitakis *et al.* focused on the NM23-H1 protein in skin lesions and both found medium/strong NM23-H1 positivity in all BCCs.[34,35] In spite of the significant expression of NM23-H1 in BCCs, its importance is not well known. However, it has been shown that NM23-H1 expression is inversely related to the metastasis status in other human carcinomas.[36] The significant NM23-H1 expression in BCCs probably contributes to their non-metastasizing feature.

One of the important results of this study is the demonstration of significant cytoplasmic NDRG1 expression in BCC, which is also supported with the qRT-PCR study. Although NDRG1 expression has not been studied in BCC previously, Cangul demonstrated that human carcinomas expressed high levels of NDRG1 compared to their normal counterpart,[37] and the prognostic importance of NDRG1 expression has been pointed out in various human tumors.[38–41] We also found a significant correlation between $NDRG1_{cyt}$ ($P = 0.001$) and E-cadherin in BCCs, and this relationship was reported in the literature in colon and prostate carcinomas.[8,42,43] Our data clearly support an E-cadherin/NDRG1 pathway in human carcinomas.

Besides NM23-H1 and NDRG1, E-cadherin expression was generally preserved in 92.7% of BCCs with relatively higher HSCORES, and qRT-PCR studies also showed no statistically significant difference from normal skin tissue. The data from the literature and our study show that E-cadherin positivity is expected in BCC, even if at reduced levels compared to the normal epidermis (Table 1).

CD82/KAI1 and AKAP12 expressions were significantly reduced or completely lost in all morphological subtypes of BCCs. We also detected downregulation of *AKAP12* mRNA in BCC. Similar to our results, the downregulation of CD82/KAI1 expression in BCCs was demonstrated before.[44] Yet, the expression pattern of AKAP12 is still not known in BCCs, a recent article pointed out that the methylation frequencies of the *AKAP12* gene were significantly higher in skin carcinomas than normal skin tissue.[45] CD82/KAI1 and AKAP12 probably have no contribution to the non-metastatic features of BCCs.

However, we believe that these proteins are interesting negative markers for BCCs, and further studies might show their role in the differential diagnosis.

One of the goals of the study was to show the correlation between the MSPs, and important clinicopathological parameters and p53 in BCCs. We found AKAP12 inversely correlated with inflammation, together with an inverse relationship between NM23-H1$_{nuc}$ and perineural invasion. Besides these expected correlations, we found that recurrences were correlated only with RHOGDI2$_{nuc}$. This result may be explained by the dual and unpredicted role of RHOGDI2 in carcinomas as proposed by Griner and Theodorescu.[14] We found only an inverse correlation between RHOGDI2$_{cyt}$ and p53. Although the relationship between p53 and RHOGDI2 has not been demonstrated previously, interaction between p53 and CD82/KAI1, another MSP, has been reported.[46–48] However, other studies have been querying this correlation, similar to our results.[49–52]

One of the major questions in this study is the contribution of MSPs to the aggressive phenotype of BCCs. We detected upregulation of NDRG1 levels in the aggressive phenotype ($P = 0.001$). Similarly, CD82/KAI levels ($P = 0.048$) were downregulated. In the literature, E-cadherin levels have been shown to be downregulated in aggressive BCCs,[11,53] but this has not been supported by other studies (Table 1). These results may show a slightly different profile of MSPs in aggressive carcinomas than non-aggressive BCCs.

In conclusion, we have demonstrated differential expression patterns for the seven MSPs in BCCs. AKAP12 and CD82/KAI1 levels were significantly reduced in BCCs. However, NM23-H1, NDRG1, and E-cadherin levels were minimally reduced, and they were generally expressed in this neoplasm group. The other markers, MKK4 and RHOGDI2, were also reduced but not lost in BCCs. Although this is a very simplified approach, preserved levels of NM23-H1, E-cadherin, and NDRG1 may contribute to the non-metastatic features of BCCs. One of our important findings is there are plenty of significant correlations among the MSPs. Data from this study might reveal possible pathways between MSPs, when combined with the current knowledge on pathways. This relationship between these MSPs warrants further biological and experimental pathway research.

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