Modern Pathology (2004) 17, 1464–1474

www.modernpathology.org

Retinoblastoma protein function and p16^{INK4a} expression in actinic keratosis, squamous cell carcinoma *in situ* and invasive squamous cell carcinoma of the skin and links between p16^{INK4a} expression and infiltrative behavior

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p16^{INK4a} is involved in many important regulatory events in the cell and the expression and function is closely associated with the retinoblastoma protein (Rb). Earlier, we have in colorectal cancer and in basal cell carcinoma showed that p16^{INK4a} is upregulated at the invasive front causing cell cycle arrest in infiltrative tumor cells via a functional Rb. This role for p16^{INK4a} as a regulator of proliferation when tumor cells infiltrate might besides a general cyclin-dependent kinase (cdk) inhibitory effect explain why p16^{INK4a} is deregulated in many tumor forms. The expression pattern of p16^{INK4a} in relation to Rb-function in squamous cancer and precancerous forms of the skin has not been fully detailed. We therefore characterized the expression of p16^{INK4a}, Rb-phosphorylation and proliferation in actinic keratosis, squamous cell carcinoma *in situ* and invasive squamous cell carcinoma with special reference to infiltrative behavior. The expression of p16^{INK4a} varied between the lesions, with weak and cytoplasmic p16^{INK4a} expression and functional Rb in actinic keratosis. Strong nuclear and cytoplasmic p16^{INK4a} expression was observed in all carcinomas *in situ* in parallel with lack of Rb-phosphorylation but high proliferation indicating a nonfunctional Rb. Invasive squamous carcinoma showed a mixed p16^{INK4a} expression pattern where some tumors had strong cytoplasmic p16^{INK4a} expression, large fraction of Rb-phosphorylated cells and high proliferation. Interestingly, despite this disability of p16^{INK4a} to inhibit proliferation there was an upregulation of cytoplasmic p16^{INK4a} in infiltrative cells compared to tumor cells towards the tumor center. A similar scenario but strong and combined nuclear and cytoplasmic p16^{INK4a} expression in infiltrative cells, was observed in other invasive squamous cancers. This suggests that the p16^{INK4a} upregulation in infiltrative cells is governed independently of the subcellular localization or of the potential to affect proliferation via Rb, and suggests a potentially proliferation independent function for p16^{INK4a} in infiltrative behavior.
Modern Pathology (2004) **17,** 1464–1474, advance online publication, 16 July 2004; doi:10.1038/modpathol.3800220

Keywords: squamous cell carcinoma; actinic keratosis; skin; p16^{INK4a}; Ki-67; phosphorylated Rb

Squamous cell carcinoma of the skin is derived from keratinocytes in the epidermal layer and is one of the most common malignancies.[1] Squamous cell carcinomas grow rather slowly, are locally invasive but rarely metastasize. It is generally believed that squamous cell carcinoma can emerge from the precancerous lesion actinic keratosis, since the skin lesion mainly occurs on chronically sun-exposed sites as squamous cell carcinoma.[2] UV-type *p53* mutations are the, so far known, most common genetic alteration in squamous cell carcinoma.[1] Another tumor suppressor gene commonly altered besides *p53* in tumors is *p16^{INK4a}*.[3] Alterations affecting *p16^{INK4a}* include small homozygous gene deletions and hypermethylation of the *p16^{INK4a}* promoter and more rarely, mutations are found in the INK4a/ARF locus.[4] In squamous cancer and associated skin lesions, the *p16^{INK4a}* gene is mutated in up to 24%.[5,6] Loss of heterozygosity of the 9p21 region has also been observed in squamous

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Received 9 March 2004; revised and accepted 24 May 2004; published online 16 July 2004

carcinomas of the skin.[2,7] The main biological function of p16INK4a is to regulate the cell cycle by binding to cyclin-dependent kinases (cdk) 4/6. This prevents the formation of an active cyclin D–cdk4/6 complex. The initial cyclin D-associated phosphorylation of the retinoblastoma protein (Rb), fundamental for cell cycle progress, is therefore inhibited resulting in a G1-arrest.[8,9] Besides cell cycle control, p16INK4a has been implicated in other processes such as senescence[10,11] and apoptosis.[12] In addition, p16INK4a has shown to reduce cell invasion,[13,14] cell spreading[15] and angiogenesis.[16]

Nonmelanoma skin cancers (NMSC) include both squamous cell carcinoma and basal cell carcinoma, and we have earlier reported that in basal cell carcinoma there is a phenotypic change from a proliferative behavior in the center of the tumor towards a less proliferative but infiltrative phenotype at the invasive front of the tumor.[17] This feature is most likely mediated by the cell cycle inhibitory protein p16INK4a via a functional Rb-pathway. We have also reported a similar phenomenon in colorectal cancer where p16INK4a upregulation was observed in small tumor clusters at the invasive front while the larger clusters had lower p16INK4a expression.[18] The smaller clusters had also lower proliferation than the larger clusters mimicking the situation in basal cell carcinoma. This indicates that p16INK4a might function as a general regulator, mediating the switch between high proliferation *vs* low proliferation and infiltrative behavior in tumor cells. Therefore, we aimed to investigate the relationship between p16INK4a and invasion as well as proliferation/phosphorylated Rb in squamous cell carcinoma of the skin and associated lesions. In summary, p16INK4a expression was observed in actinic keratosis, carcinoma *in situ* and invasive squamous cell carcinoma but in varying amounts. The expression of p16INK4a did not affect the proliferation of the cells in squamous cell carcinoma *in situ* and invasive squamous cell carcinoma indicating that disruption of the Rb-pathway is a common event in these tumors. Further, increased p16INK4a expression at the invasive front was observed in invasive squamous cell carcinomas, in a similar manner to basal cell carcinoma, which might indicate a common functional role of p16INK4a in squamous cell carcinoma and basal cell carcinoma invasion.

Materials and methods

Immunohistochemistry and Tumor Materials

A total of 35 tumors, 10 actinic keratoses, 12 squamous cell carcinomas *in situ* and 13 invasive squamous cell carcinomas, were immunohistochemically stained and evaluated for p16INK4a, Ki-67 and phosphorylated Rb in serial sections. Six tumors were also evaluated using double staining with p16INK4a and phospho-Rb antibodies and eight tumors were double stained with p16INK4a and Ki-67. For comparison, six Merkel cell carcinomas were also stained for p16INK4a, Ki-67 and phosphorylated Rb.

Paraffin sections of 4 μm were deparaffinized using xylene and rehydrated using descending concentrations of ethanol according to the standard protocol. For the antibodies, anti-human p16INK4a (1:200, BD PharMingen, San Jose, CA, USA), anti-human Ki-67 antigen (1:200, Dako A/S, Glostrup, Denmark), anti-human MNF116 (1:400, Dako) and anti-human phospho-Rb (1:150, Cell Signaling Technology, Beverly, MA, USA), antigen retrieval was achieved by microwave heating in 10 mM citrate buffer, pH 6.0. Single and double staining were performed in a DakoTechmate 500 (Dako) according to the manufacturer's instructions.

Cell Line Array

The breast cancer cell lines MDA-MB-468, T-47D and MCF7 (American Type Culture Collection, Rockville, MD, USA) were used as phospho-Rb antibody control. MDA-MB-468 and MCF7 were grown in RPMI 1640 supplemented to contain 10% FCS and 1 mM sodiumpyruvate. T-47D was grown in DMEM supplemented to contain 10% FCS, 10 mM HEPES and 0.2 U/ml insulin. Cells were harvested, fixed in 1 ml 4% paraformaldehyde for 30 min and stained by adding Mayer's hematoxylin. The paraformaldehyde was removed, 1 ml 70% ethanol was added and cells were incubated overnight. The cells were dehydrated using increasing concentrations of ethanol and finally xylene. After dehydration, the cells were embedded in paraffin and arranged in a cell line array.

Western Blotting

Cultured cells were washed in PBS, harvested by scraping, centrifuged at 250 *g* for 5 min and frozen at -80°C overnight. Tumors were collected immediately after surgery and frozen in 2-methylbutane using liquid nitrogen as freezing agent. Cell pellets and areas corresponding to neoplasia were homogenized, and the tumor extracts were sonicated 2×15 s, in lysis buffer containing 50 mM Tris-HCl pH 7.5, 0.5% (v/v) NP-40, 0.5% (w/v) sodiumdeoxycholate, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM NaF and 0.1 mg/ml PMSF supplemented with the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany). The protein lysates were centrifuged at 19 000 *g* for 30 min and the supernatants were collected. For detecting p16INK4a, 40 μg of the protein extracts were run on a 12% polyacrylamide gel and for phospho-Rb, a 5% polyacrylamide gel was used. The proteins were transferred on to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were probed with anti-human

p16INK4a (1:500; BD PharMingen), anti-human phospho-Rb (1:500, Cell Signaling Technology) or anti-human actin (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies followed by peroxidase-conjugated anti-mouse (1:5000, Amersham Biosciences), anti-rabbit (1:5000, Amersham Biosciences) or anti-goat (1:5000, Sigma, St Louis, MO, USA) antibodies. The proteins were detected using enhanced chemiluminescence detection system plus (ECL + plus) reagent (Amersham Biosciences) according to the manufacturer's instructions and exposed on ECL Hyper Film (Amersham Biosciences).

Results

p16INK4a protein expression was evaluated by immunohistochemistry in 35 skin lesions of which 10 were actinic keratoses, 12 squamous cell carcinomas *in situ* and 13 invasive squamous cell carcinomas. All lesions were p16INK4a positive with highly variable staining intensities and fraction of positive tumor cells. The subcellular localization of the p16INK4a staining varied considerably and was therefore also delineated (Table 1). Actinic keratosis had in general rather weak p16INK4a staining and the staining was mainly cytoplasmic with occasional positive nuclei in some lesions (Figure 1a and b). Out of 12 squamous cell carcinomas *in situ*, 11 showed a strong nuclear and cytoplasmic p16INK4a staining (Figure 1c and d), which was in sharp contrast to surrounding normal keratinocytes. The remaining squamous cell carcinoma *in situ* also had a strong p16INK4a staining although with a mixed pattern of areas with cytoplasmic staining and areas with both nuclear and cytoplasmic staining. Of the invasive squamous cell carcinomas, nine had only cytoplasmic p16INK4a staining (Figure 1e and f) and four combined nuclear and cytoplasmic staining (Figure 1g and h) (Table 2). The intensity of the p16INK4a staining in invasive squamous cell carcinomas varied from weak staining in some tumors to very strong staining in others. To further validate the p16INK4a staining, Western blotting was performed using protein extracts prepared from frozen actinic keratoses, squamous cell carcinomas *in situ* and invasive squamous cell carcinomas with available p16INK4a immunohistochemistry data. The Western blot produced a 16 kDa band with varying intensities corresponding to p16INK4a in all tested tumors (Figure 2) confirming the immunohistochemistry data.

To investigate whether the observed p16INK4a was functional and affected the Rb-pathway, the degree of Rb-phosphorylation in the different skin lesions was analyzed by immunohistochemistry, using an antibody specifically recognizing phosphorylation of serines 807 and 811 on Rb, in sections serial to the p16INK4a staining. Colocalization of p16INK4a and phosphorylated Rb was additionally confirmed in six tumors by double staining. To verify the specificity of the antibody, protein extracts and a cell line array, mimicking the settings for the paraffin-embedded tumors, were simultaneously prepared from three different cell lines. The two cell lines T-47D and MCF7, known to have a functional Rb, showed by immunohistochemistry an intense nuclear staining with the phospho-Rb antibody and produced a strong band of 110 kDa corresponding to phosphorylated Rb by Western blot analysis. In contrast, the Rb-inactivated cell line MDA-MB-468 was negative in both analyses clearly validating the specificity of the phosphospecific Rb antibody in analyses of formalin-fixed materials (Figure 3).

All of the 12 investigated squamous cell carcinomas *in situ* showed as earlier presented high p16INK4a expression and all these tumors were, except for the one tumor with mixed p16INK4a pattern, completely negative in the analyses of Rb-phosphorylation (Figure 4a and c). For the invasive squamous cell carcinomas with p16INK4a expression only in the cytoplasm (Figure 5a and c), colocalization between p16INK4a and phosphorylated Rb was observed in all of the nine tumors (Figure 5e), although there was a heterogenous expression of phosphorylated Rb in one tumor. The invasive squamous cell carcinomas with both nuclear and cytoplasmic p16INK4a staining (Figure 6a and c) were negative for phosphorylated Rb (Figure 6e) (Table 2). In all actinic keratoses phosphorylated Rb was observed.

To further delineate the functional consequences and ultimate effect on proliferation in the different skin lesions associated with the varying p16INK4a expression in the nucleus and the cytoplasm, we characterized the presence of the proliferation marker Ki-67 in the tumors using sections serial to the p16INK4a staining. Colocalization of p16INK4a and Ki-67 was confirmed in eight tumors by double staining. The actinic keratoses were in general low

Table 1 Evaluation of p16INK4a expression in cancerous and precancerous lesions of the skin

Lesion	*p16INK4a localization*	*p16INK4a staining*
Actinic keratosis	N+C	0
	C	10[a]
	Negative	0
Carcinoma *in situ*	N+C	12[b]
	C	0
	Negative	0
Invasive squamous cell carcinoma	N+C	4[b]
	C	9
	Negative	0

Nuclear and cytoplasmic staining (N+C), cytoplasmic staining only (C).

[a]In four actinic keratoses, a few scattered cells with nuclear staining was seen.

[b]One squamous lesion had a mixed pattern of both nuclear and cytoplasmic as well as only cytoplasmic staining.

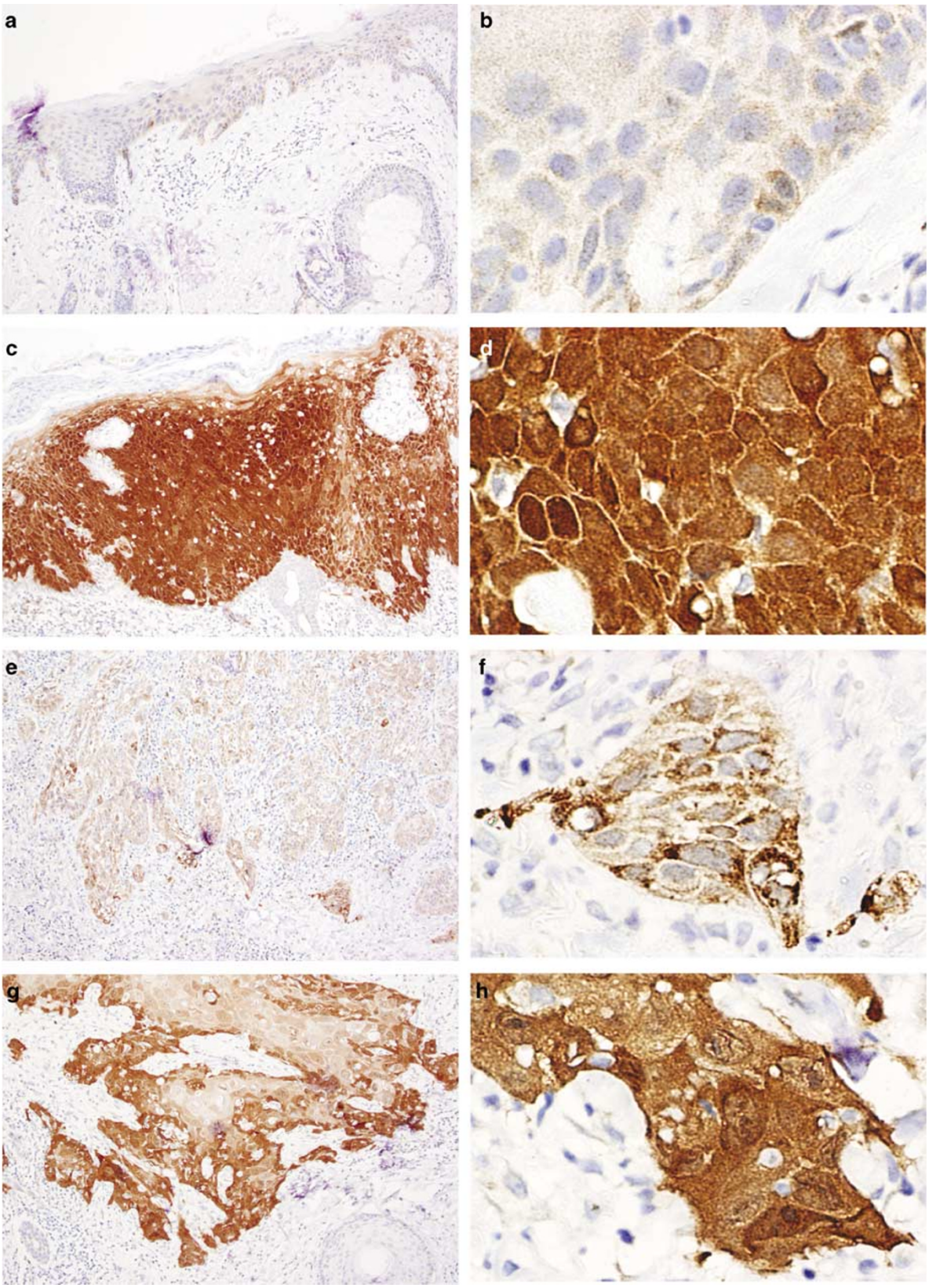

Figure 1 $p16^{INK4a}$ staining in precancerous and cancerous forms of squamous cell carcinoma of the skin. Actinic keratosis with cytoplasmic staining (**a**, **b**), $p16^{INK4a}$ staining in squamous cell carcinoma *in situ* showing full-thickness staining (**c**, **d**), invasive squamous cell carcinoma with cytoplasmic staining only (**e**, **f**) and both nuclear and cytoplasmic staining (**g**, **h**).

Table 2 Ki-67 and phospho-Rb expression in p16^{INK4a}-positive tumor areas/cells as well as localization of p16^{INK4a} in invasive squamous cell carcinomas

Invasive squamous cell carcinoma number	*p16^{INK4a} subcellular localization*	*Ki-67*	*Phospho-Rb*	*Localization of p16^{INK4a}-positive cells towards the invasive front*	*Differentiating cells in the center of the tumor*
1	C	+	+	Yes	Yes
2	C	+	+	No	Yes
3	C	+	+[b]	No	Yes
4	C	+	+	Yes	No
5	N+C	+	−	Yes	Yes
6	C	+	+	Yes	No
7	N+C[a]	−	−	Yes	No
	C[a]	+	+	Yes	No
8	N+C	+	−	Yes	Yes
9	C	+	+	Yes	Yes
10	C	+	+	Yes	Yes
11	C	+	+	Yes	No
12	C	+	+	Yes	Yes
13	N+C	+	−	No	No

Nuclear and cytoplasmic staining (N+C), cytoplasmic staining only (C).
[a]Tumor with two different areas of p16^{INK4a} expression.
[b]Less phospho-Rb-positive than Ki-67-positive tumor cells.

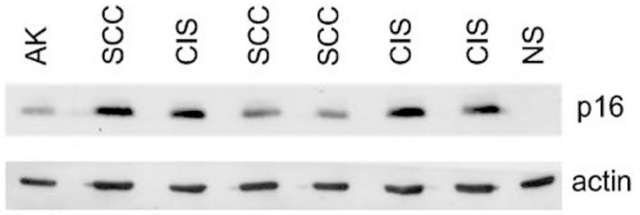

Figure 2 Western blotting showing p16^{INK4a} expression in actinic keratosis (AK), squamous cell carcinoma *in situ* (CIS) and invasive squamous cell carcinoma (SCC) of the skin. Protein extract from normal skin (NS) was used as p16^{INK4a}-negative control and actin was used as loading control.

proliferative with only few Ki-67-positive cells towards the base of the epidermis and analyzing Rb-phosphorylation, there was nevertheless an overlap between weak cytoplasmic p16^{INK4a} expression and presence of proliferation. In all carcinoma *in situ* lesions, there was a high fraction of Ki-67-positive cells despite high p16^{INK4a} expression (Figure 4a and b). In all but one of the invasive squamous cell carcinomas, regardless of the subcellular localization of p16^{INK4a}, Ki-67 was present in the p16^{INK4a}-positive areas of the tumors (Figures 5a–d and 6a–d and Table 2).

As earlier shown in basal cell carcinoma[17] and in colorectal cancer,[18] p16^{INK4a} is expressed in infiltrative tumor cells matching a decrease in proliferation via an intact Rb-pathway. The relation between p16^{INK4a} and infiltrative growth in squamous cell carcinomas of the skin where p16^{INK4a}, via Rb, cannot fulfill its growth inhibitory function is nevertheless not clear. We therefore delineated the p16^{INK4a} protein expression at the invasive front and in more central parts of the invasive squamous cell carcinomas. In 10 out of the 13 invasive squamous cell carcinomas, p16^{INK4a} protein expression was higher in tumor cells located towards the invasive front as well as in small tumor clusters compared to central parts of the tumors (Table 2). Surprisingly, p16^{INK4a} seemed to be expressed in infiltrating tumor cells despite the lack of a functional Rb-pathway. In one of the invasive lesions we also noticed localization of p16^{INK4a} towards ulceration of the skin. In addition to the increased p16^{INK4a} expression at the invasive front in squamous cell carcinoma we also observed a decrease in p16^{INK4a} expression in tumor cells that were more differentiated and close to areas with keratin formations (Figure 7) (Table 2).

To compare squamous cell carcinoma with a more aggressive tumor type, we also investigated the p16^{INK4a} expression pattern in six Merkel cell carcinomas, a neuroendocrine tumor of the skin. These tumors expressed high levels of p16^{INK4a} in the nucleus and the cytoplasm throughout the tumor without any clear localization towards the invasive front, although small tumor clusters also expressed high levels of p16^{INK4a}. All tumors were highly proliferative and phosphorylated Rb was completely absent or present in a much lower fraction than Ki-67, which may suggest a defect Rb-pathway in Merkel cell carcinoma although the results were not as clear as in invasive and *in situ* squamous cell carcinoma (data not shown).

Discussion

As shown in this study, p16^{INK4a} was commonly expressed in precancerous and cancerous skin lesions, but there was a marked difference in expression pattern and subcellular localization of the protein. In all 35 skin lesions, we noted a

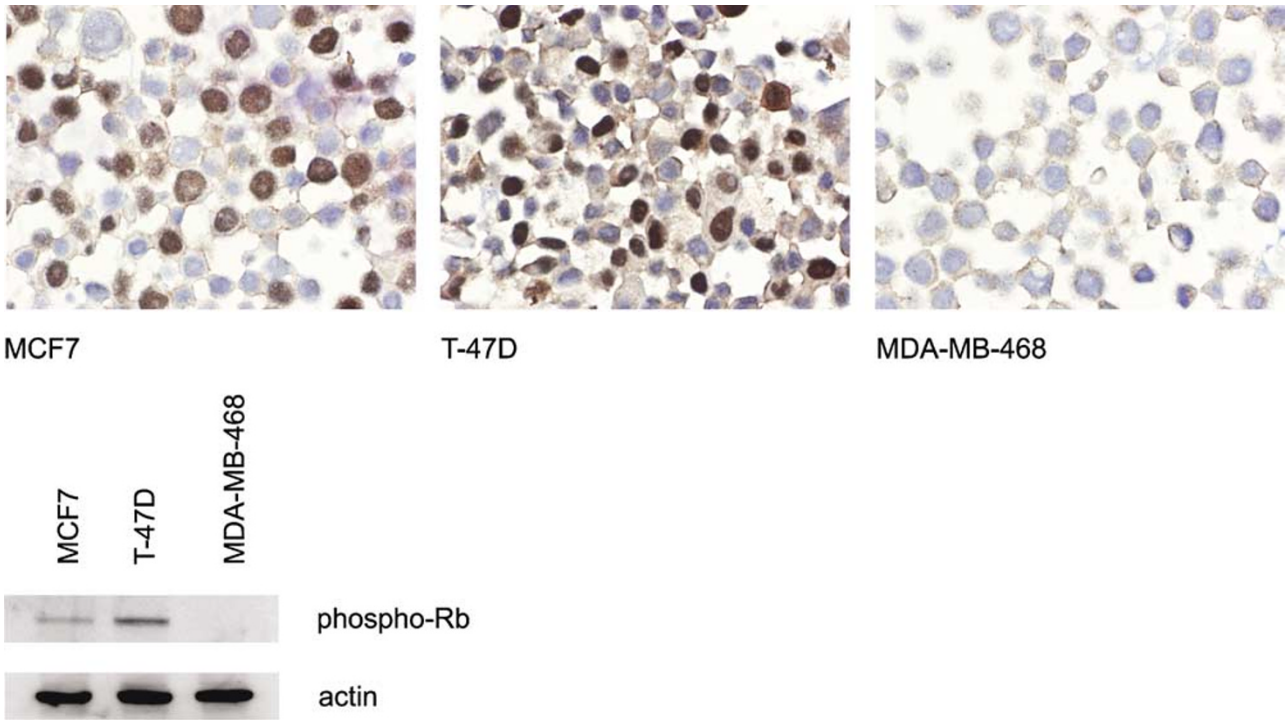

Figure 3 Verification of the phospho-Rb antibody using Western blotting and immunohistochemistry. The cell lines MCF7 and T-47D were used as positive controls and gave strong bands on the Western blot. Nuclear staining was seen in both cell lines using immunohistochemistry. The Rb-inactivated cell line MDA-MB-468 produced no nuclear staining using immunohistochemistry and no band was seen on the Western blot.

p16^{INK4a} expression although with variations between the different lesions and also between different tumors within the same group. These results are in agreement with previous reports stating that p16^{INK4a} expression is common in actinic keratosis[1,2] and in squamous cell carcinoma *in situ*.[1] However, the expression of p16^{INK4a} in invasive squamous cell carcinoma has been argued. In two recent reports, in which immunohistochemistry was used, p16^{INK4a} expression was observed in a few invasive squamous cell carcinomas only,[2,19] whereas others have reported that all invasive squamous cell carcinomas were p16^{INK4a} positive.[1] Our results based on immunohistochemistry and validated by Western blotting of protein extracts from tumor materials, indicate that p16^{INK4a} is commonly expressed in invasive squamous cell carcinoma although apparently inactivated in a fraction of the tumors as observed by the localization to the cytoplasm in tumor cells. Cytoplasmic p16^{INK4a} staining has been observed in cell lines and tumors with homozygous deletion of the *p16^{INK4a}* locus and therefore been considered unspecific.[20] However, using Western blotting we observed a band corresponding to p16^{INK4a} in a squamous cell carcinoma exhibiting only cytoplasmic p16^{INK4a} staining by immunohistochemistry, which indicates a true p16^{INK4a} staining in the cytoplasm.

Using a phosphospecific Rb antibody the presence of functional Rb was evaluated in the skin lesions. As mentioned above, there was a clear cytoplasmic localization of p16^{INK4a} in some of the invasive squamous carcinomas, but simultaneously these tumors were highly proliferative and showed widespread phosphorylation of Rb. This suggested that Rb was functional but that the Rb-pathway in general seemed to be disrupted by a delocalization of p16^{INK4a} to the cytoplasm. The reason why p16^{INK4a} was localized to the cytoplasm is not clear but could be caused by a mutation prohibiting the translocation to the nucleus. It has previously been reported that 14% of squamous cell carcinomas have intragenic mutations or deletions in exon 2 of the INK4a/ARF locus affecting both *p16^{INK4a}* and *p14^{ARF}*.[6] In another report, exon 2 of the INK4a/ARF locus was mutated in 24% of squamous lesions.[5] It is though apparent, that the cytoplasmic p16^{INK4a} did not inhibit the phosphorylation of Rb and consequently did not affect proliferation. In actinic keratosis, it is unclear whether the Rb-pathway was functional or not due to the cytoplasmic localization of p16^{INK4a} in combination with low proliferation.

In the tumors with a strong nuclear as well as cytoplasmic localization of p16^{INK4a}, as observed in squamous cell carcinomas *in situ* and in some invasive squamous cell carcinomas, no Rb-phosphorylation was detected suggesting either that p16^{INK4a} fulfilled its function or that Rb was inactivated. This could be further clarified by taking into account proliferation, where proliferative arrest is the ultimate end result for p16^{INK4a} and Rb, and

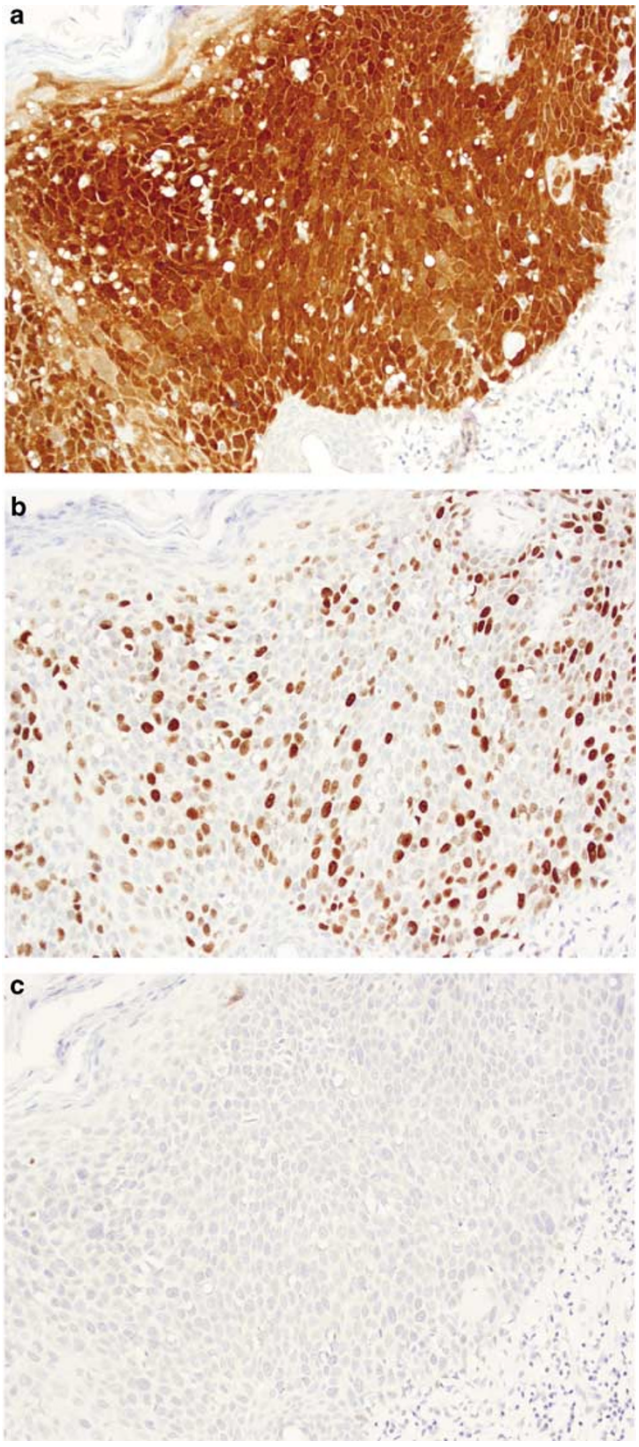

Figure 4 Serial sections of a squamous cell carcinoma *in situ* stained with p16INK4a (**a**), Ki-67 (**b**) and phospho-Rb (**c**) antibodies.

these tumors were still proliferating despite large amounts of p16INK4a also in the nucleus. The combination of high proliferation, lack of Rb-phosphorylation and high p16INK4a expression therefore indicated the presence of an inactive Rb-pathway. It is well known that human papilloma virus (HPV), which inactivates p53 and Rb, is involved in the development of cervical squamous carcinoma and the Rb-inactivation probably contributes to the high p16INK4a expression in cervical cancer.[21–23] The role of HPV in NMSC has been debated and extensively studied but remains elusive.[22] The similarities between squamous cell carcinomas of the skin and cervix are, nevertheless, profound and both the Rb-inactivation and high p16INK4a expression support a common and probably HPV-associated etiology.

Regarding upregulation of p16INK4a in infiltrative squamous cell carcinoma tumor cells, we observed a clear increase of p16INK4a in small infiltrative clusters as well as towards the invasive front of squamous cell carcinomas. The difference was although not as obvious as in basal cell carcinoma where there is a massive upregulation from undetectable levels in the tumor center to high expression of p16INK4a in infiltrative areas. In parallel, we observed a decrease in proliferative activity as well as Rb-phosphorylation in the same areas in basal cell carcinoma outlining the functional consequences for p16INK4a upregulation.[17] We have earlier also reported similar findings in colorectal cancers with high p16INK4a expression and low proliferation in small invasive tumor clusters at the invasive margin of the tumors.[18] In Merkel cell carcinoma there was no obvious localization of p16INK4a towards the invasive front even though small tumor clusters also expressed p16INK4a. However, these tumors are clearly different from keratinocyte-derived tumors and might behave differently. Both basal cell carcinoma and the colorectal cancers with p16INK4a upregulation were Rb-functional and p16INK4a seemed to be involved in the switch from a proliferative to a nonproliferative state and possibly in the invasive behavior in these tumors. Even though p16INK4a was not able to exert its function as an inhibitor of proliferation via Rb and the subcellular localization of p16INK4a seemed disrupted in some squamous lesions, p16INK4a was still upregulated at the invasive front. This could indicate that p16INK4a is involved in regulating infiltrative behavior independently of Rb. p16INK4a has been suggested to inhibit tumor cell invasion and migration,[13–15,24] processes that involve many cytoplasmic proteins. Cdk6 has shown to be localized to the spreading edge of human fibroblasts and could potentially be a target for inhibition of cell spreading by G1-associated kinase inhibitors.[15] In support of our results, it has also been reported that keratinocytes migrating towards a wound upregulate p16INK4a and proliferation is at the same time decreased, linking p16INK4a to wound-healing processes.[19] In one squamous cell carcinoma, we also observed p16INK4a upregulation at the edge of an ulcer. Similar mechanisms regarding p16INK4a upregulation in infiltrative tumor cells as observed in basal cell carcinoma, colorectal cancer and now in squamous cell carcinoma might therefore be natural processes used in many cellular events that include

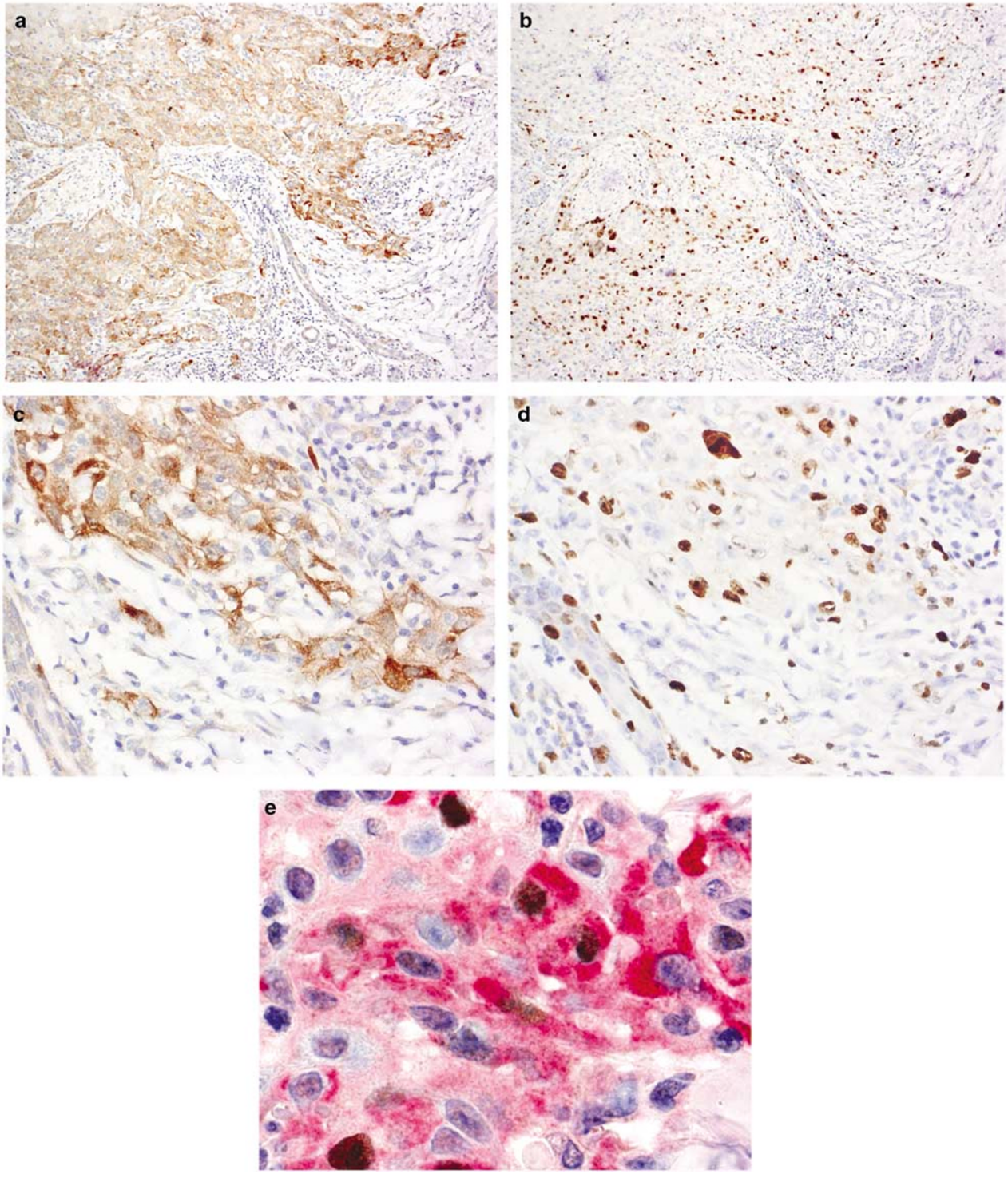

Figure 5 Serial sections of an invasive squamous cell carcinoma stained with p16^{INK4a}, Ki-67 and phospho-Rb antibodies. Cytoplasmic p16^{INK4a} staining (**a**, **c**) and the corresponding Ki-67 staining (**b**, **d**) showing high-proliferative activity in invasive squamous cell carcinoma. Note the increased expression of p16^{INK4a} towards the invasive front. Double staining (**e**) showing phosphorylated Rb (brown) in the p16^{INK4a} (red)-positive cells.

migration of proliferative cell types where wound healing is one example.

The expression of p16^{INK4a} was further decreased in squamous cell carcinoma areas with keratin differentiation. This decrease may indicate a function for p16^{INK4a} in keeping the cell in a nondifferentiated state or alternatively could the loss of p16^{INK4a} be a consequence of the differentiation

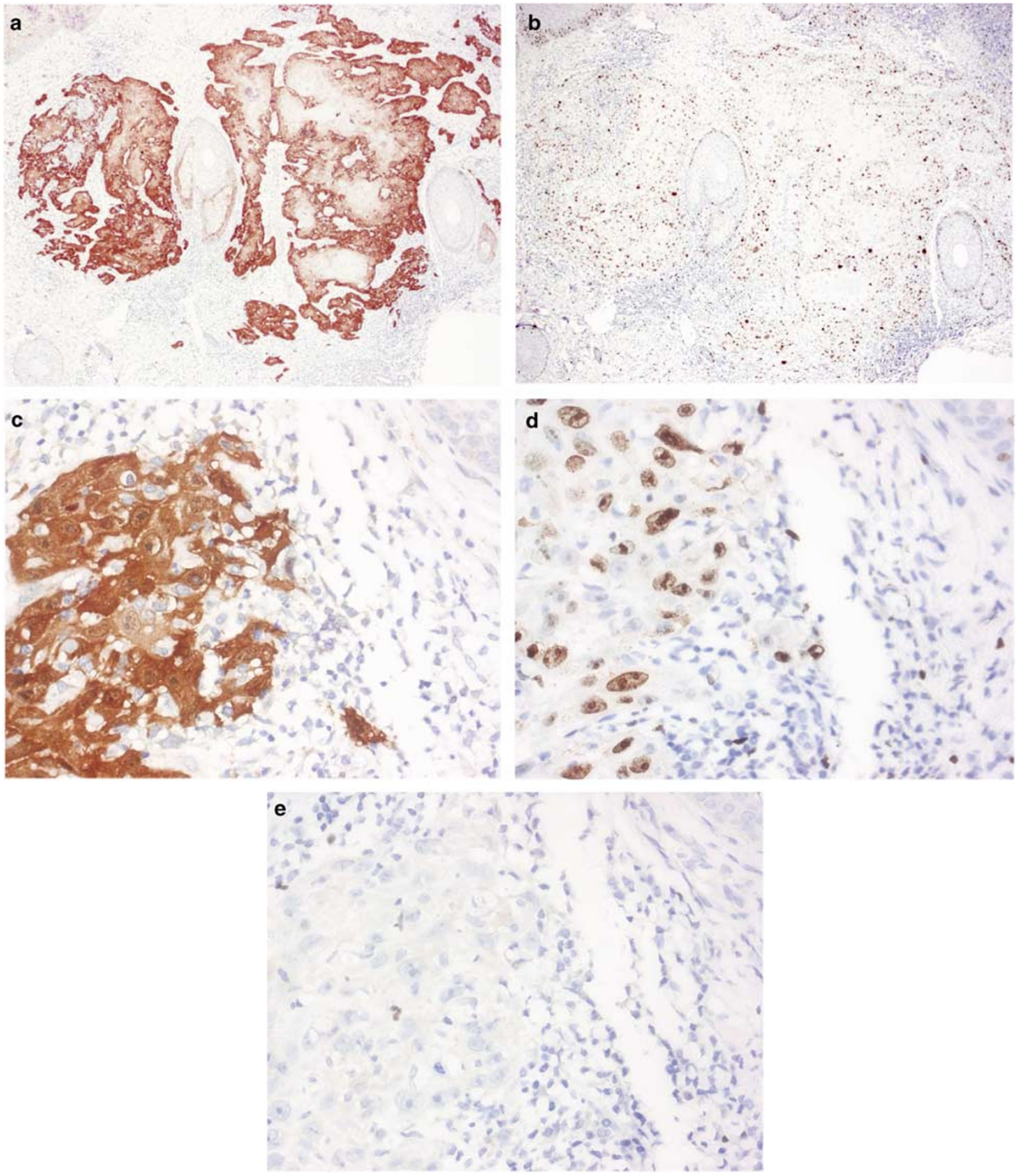

Figure 6 Serial sections of an invasive squamous cell carcinoma stained with p16^{INK4a}, Ki-67 and phospho-Rb antibodies. Nuclear and cytoplasmic p16^{INK4a} staining (**a**, **c**) and the corresponding Ki-67 staining (**b**, **d**) showing high-proliferative activity in invasive squamous cell carcinoma. Note the increased expression of p16^{INK4a} towards the invasive front and the decreased expression in differentiating cells in the center of the tumor. Single staining of phosphorylated Rb (**e**) in a p16^{INK4a} and Ki-67 high area of the tumor.

process. p16^{INK4a} has earlier been shown to be associated with induction of squamous cell differentiation in normal human epidermal keratinocytes, but overexpression of p16^{INK4a} did not induce differentiation in the same cells.[25] In a human trophoblast cell line, p16^{INK4a} is upregulated during TGFβ1-induced differentiation[26] and in K562 cells forced p16^{INK4a} expression promoted erythroid differentiation,[27] suggesting that p16^{INK4a} is involved in differentiation in different cell types.

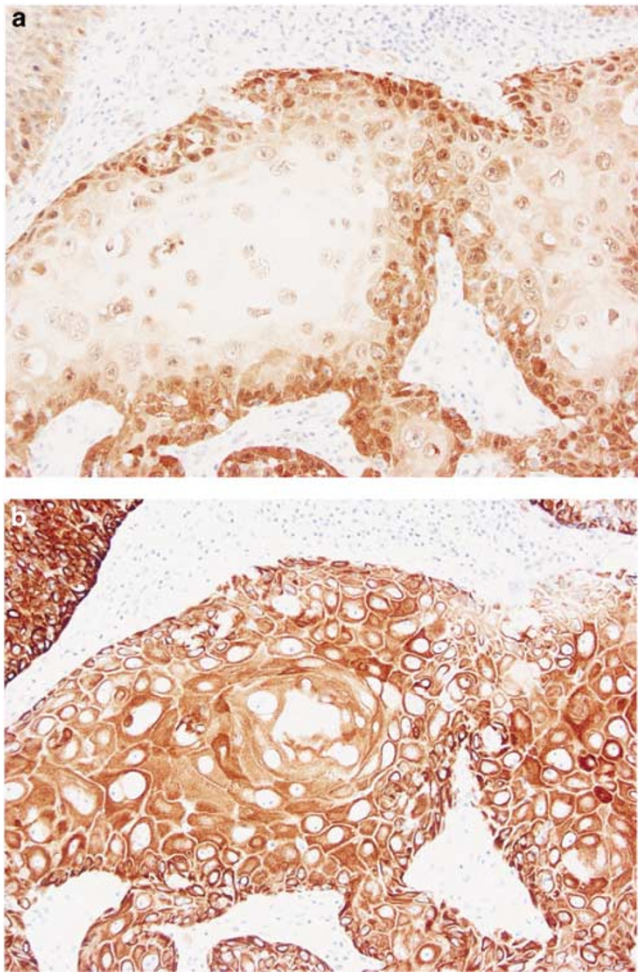

Figure 7 p16^{INK4a} staining (**a**) of an invasive squamous cell carcinoma. Note the reduced p16^{INK4a} expression in areas with keratin differentiation. MNF116 (**b**) was used as a control for presence of keratinocytes.

In summary, we have shown that p16^{INK4a} is expressed in cancerous and precancerous lesions of the skin and by specifically studying phosphorylation of Rb in parallel with p16^{INK4a} and proliferation shown that both squamous cell carcinoma *in situ* and invasive squamous cell carcinoma have a nonfunctional Rb-pathway but with partially contrasting etiologies. The upregulation of p16^{INK4a} towards the invasive front of invasive squamous cell carcinomas despite an inactive Rb-pathway also supports that p16^{INK4a} could be involved in infiltrative processes independent of proliferation effects.

Acknowledgements

We thank Elise Nilsson for excellent technical assistance. We also thank the personnel at the Department of Plastic and Reconstructive Surgery, Malmö University Hospital, Sweden and all the patients who participated in this study. This research was supported by grants from the Swedish Cancer Society, Gunnar, Arvid and Elisabeth Nilsson Cancer Foundation, Lund University Research Funds, and Malmö University Hospital Research and Cancer Funds.

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