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Progression of actinic keratosis to squamous cell carcinoma of the skin correlates with deletion of the 9p21 region encoding the $p16^{INK4a}$ tumor suppressor

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Abstract

Actinic keratoses (AKs) are pre-neoplastic lesions that can develop into squamous cell carcinomas (SCCs) of the skin. Often AK and SCC have commonly altered p53. A status of another tumor suppressor, the $p16^{INK4a}$, was reported for SCC but not for AK. A comparative study of SCC and AK human samples by loss of heterozygosity (LOH) analysis determined that the $p16^{INK4a/ARF}$ locus is less frequently altered in AKs than in SCCs. These LOH data highly correlated with immunohistochemical findings demonstrating the presence of $p16^{INK4a}$ in the AK skin samples but its absence in SCC lesions. Our results imply that progression of AK into SCC may involve inactivation of $p16^{INK4a}$.

Keywords: Squamous cell carcinoma; Actinic keratosis; $p16^{INK4a}$; p53; Loss of heterozygosity

1. Introduction

Squamous cell carcinomas (SCCs) of the skin are the most prevalent keratinocyte-derived tumors, second to basal cell carcinomas (BCCs) [1].

Typically, SCCs are slow growing, locally invasive and poorly metastasizing tumors, often developing within pre-neoplastic lesions of actinic keratosis (AK). Indeed, 97% of SCCs were associated with contiguous AKs [2]. Based on epidemiological, clinical, histopathological and molecular studies, AK is considered an early step in SCC development (for review see Ref. [3]). Both AK and SCC, in most cases, develop in the sun-exposed areas and affect patients predominantly with fair skin in their sixties, or young people predisposed to cancer. UV radiation (UVR) is a prevailing factor implicated in the etiology of SCCs and AKs as evidenced by the high frequency of UV-related mutations in the p53 tumor suppressor gene identified in SCCs and AKs [4]. These findings emphasize the importance of the p53-dependent mechanism in skin tumor development.

In addition to the disrupted p53-dependent mechanism, development of SCC also involves dysregulation of the pRB-controlled pathway of the cell cycle progression including inactivation of $p16^{INK4a}$. $p16^{INK4a}$ is a tumor suppressor encoded by the CDKN2A gene in the 9p21 region. It functions as

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an inhibitor of the D-type cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) involved in the regulation of the pRB activity. $p16^{INK4a}$ by inhibiting the pRB phosphorylation suppresses the entry of the G1 cells into the S phase of the cell cycle (for review see Ref. [5]). Consequently, inactivation of $p16^{INK4a}$ either at the level of gene expression (point mutation, deletion or promoter hypermethylation) or protein structure and function disrupts the cell cycle control and leads to tumor development.

Knockout mice studies and screening of human tumors for genetic and functional abnormalities clearly identify the INK4a/ARF locus as an important target in cancer [6].

The candidacy of $p16^{INK4a}$ as a critical factor affected in SCC is supported by findings that inactivation of $p16^{INK4a}$ is a frequent event in esophageal cancer [7] and in head and neck carcinomas [8], both of the same epidermoid histological type as SCC. Loss of heterozygosity (LOH) in the 9p region at high frequency in SCC [9] further supports the implication of $p16^{INK4a}$ in SCC development.

In this study we investigated the integrity of the 9p21 region containing the CDKN2A gene and correlated it with the $p16^{INK4a}$ expression in AK and SCC of the skin. We determined that unlike SCC, the AK lesions are relatively free of genetic alterations in the 9p21 region. Consequently, the $p16^{INK4a}$ expression, absent in most SCC, is not affected in AK cells. Since AK lesions often progress into SCCs, our findings suggest that a molecular event triggering this progression may involve inactivation of the $p16^{INK4a}$ tumor suppressor in AK keratinocytes carrying already mutated p53.

2. Materials and methods

2.1. Tissue samples and cells

A total of 48 SCC (28 women and 20 men) and 27 AK (13 women and 14 men) skin samples were used in this study. Samples of SCC and AK used in LOH and in immunohistochemical analysis were selected using the Lille Dermatopathology sample collection and database. All the different cases were examined by the same dermatopathologist to standardize diagnosis. The mean age of the SCC patients was 69.3 years and of the AK patients 62.3 years. Due to limitations in the availability of biological material, tissue samples for LOH analysis were different than those used for immunohistochemical studies. For LOH analysis, we used 38 SCCs and 15 AKs and for immunohistochemistry ten SCCs and 12 AKs.

For Western blotting, lysates were prepared from one SCC and one typical AK lesion. Normal keratinocytes prepared from foreskin tissue were used as a control. Foreskin was obtained after circumcision. Primary keratinocyte cultures were maintained in KGM-2 defined medium (Bio Whittaker).

All tissue sample collection was performed in compliance with the Ethical Committee at CHRU LILLE established for research on human subjects.

2.2. LOH analysis

DNA for PCR was isolated from the case-matching control and tumor paraffin-embedded tissue samples by proteinase K digestion and phenol-chloroform extraction. To detect LOH affecting $p16^{INK4a/ARF}$ and p53 loci, four microsatellite markers were used, two for each locus; D9S162, D9S171 to detect LOH in the 9p21 region and D17S938, D17S786 in the 17p13.1 region. PCR was performed in a 25 μl reaction using 40 ng of DNA, 25 pmol of each primer, 1.5 mM $MgCl_2$, 2 μl deoxynucleotide triphosphates and 1 unit of TAQ DNA polymerase (AmpliTaq Gold™, Perkin-Elmer). For each of the microsatellite markers a fluorescent primer was annealed at the 5′ extremity of the DNA sequence. Amplification consisted of 35 cycles of 45 s at 94°C, 45 s at 50°C, 1 min and 30 s at 72°C and a final 5 min extension phase at 72°C. The PCR products were analyzed on 6% polyacrylamide denaturing gels using an automated fluorescent DNA sequencer (Applied Biosystems, model 377 XL). The PCR products of the four microsatellites were mixed according to their size and type of fluorescent label. Each sample containing 1 μl of the mixed PCR products, 2.4 μl formamide, 0.8 μl of a fluorescent size marker (400 HD ROX™, Perkin-Elmer), and 0.8 μl of loading buffer was denatured for 5 min at 95°C and loaded into five wells of the pre-warmed gel. The gel was electrophoresed at 3000 W for 3.5 h at 51°C using an ABI automated sequencer. The fluorescence was detected by laser scanning. Collected data were

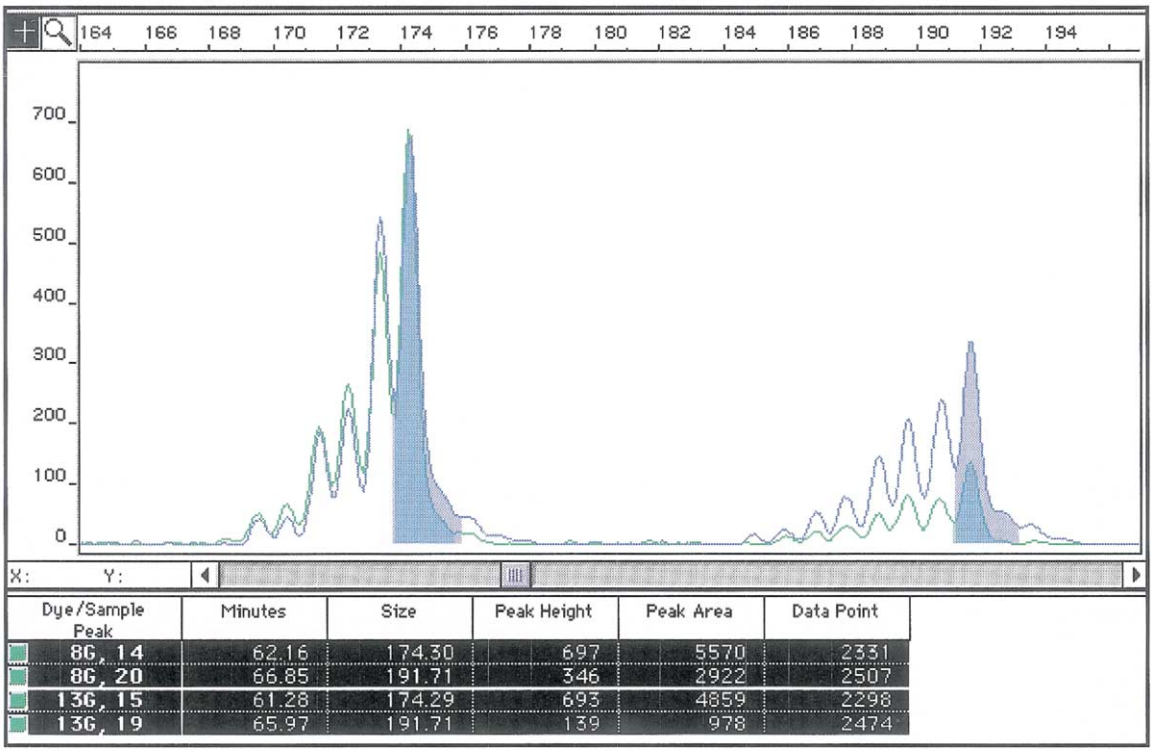


Fig. 1. The size (height and area) of each fluorescent peak was quantitated and compared with the profile of the case-matched normal DNA sample to determine hemizygosity (one peak) or heterozygosity (two peaks) of the DNA sample. For a heterozygous sample, to determine an allele loss, the peak area value for each of the two alleles in the paired normal and tumor samples was calculated according to the formula described in Section 2. The blue color corresponds to normal and the green color to tumor DNA.

stored and analyzed using the Genescan Collection software (Applied Biosystems, Foster City, CA).

The size (height and area) of each fluorescent peak was quantified and compared with the profile of the paired normal DNA sample to determine the hemizygosity (one peak) or heterozygosity (two peaks) of the DNA sample. For a heterozygous sample, to determine an allele loss, the peak area value for each of the two alleles in the paired normal and tumor samples was calculated according to the formula: (T1:T2)/(N1:N2) as described by Solomon et al. [10], where T1 and N1 are the values of the shorter allele of tumor (T) and normal (N) DNA and T2 and N2 are the values of the respective longer allele [10–12]. Theoretically, a complete allele loss should give the ratio of 0.00, but because tumor samples are usually contaminated with normal or inflammatory cells in various proportions, the value of 0.6 was selected to determine allele loss [13,14]. If this value was higher than 0.6, LOH was interpreted as negative. If the value was above 1.00 the equation was converted to 1/[(T1:T2):(N1:N2)] to get results in the range of 0.00–1.00.

2.3. Immunohistochemistry

Five micrometer tissue sections were cut and slides were baked overnight at 60°C. Sections were deparaffinized in xylen and rehydrated in gradually decreasing ethanol concentrations. Following water wash, the slides were incubated in a sodium citrate buffer (pH 6.0) for 35 min at 95°C. After a progressive decrease of temperature, sections were washed twice in phosphate-buffered saline (PBS) (pH 7.0). To detect $p16^{INK4a}$ a mouse monoclonal antibody clone G175-405 (PharMingen) was used. Staining was performed using a Histostain-plus Kit (Zymed) according to the manufacturer's instructions.

Table 1
Summary of microsatellite instability and LOH in the 17p13.1 region in 38 patients with SCC

Marker	Case number[a]																																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
D17S938	⊗	●	●	●	⊗	●	●	⊗	●	○	◒	●	⊗	○	●	●	⊗	⊗	●	●	⊗	⊗	⊗	○	●	●	○	⊗	●	●	●	●	◒	●	⊗	⊗	⊗	○
D17S786	●	○	⊗	●	●	⊗	○	⊗	●	⊗	●	●	⊗	●	●	○	⊗	●	⊗	●	⊗	●	●	○	○	●	●	●	⊗	⊗	○	●	●	⊗	●	○	●	●

[a] ●, LOH; ◒, microsatellite instability; ○, homozygous; ⊗, heterozygous and retention cases.

Table 2
Summary of microsatellite instability and LOH in the 17p13.1 region in 15 patients with AK

Marker	Case number[a]														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D17S938	⊗	○	⊗	●	⊗	○	●	●	○	⊗	●	○	○	⊗	●
D17S786	⊗	⊗	●	○	●	⊗	●	○	⊗	●	⊗	●	○	○	⊗

[a] ●, LOH; ○, homozygous; ⊗, heterozygous and retention cases.

2.4. Western blotting

Whole cell lysates from cultured primary neonatal keratinocytes and SCC and AK tissues were prepared in 1 ml of RIPA lysis buffer as described previously [15]. Total protein (100 μg) was electroblotted onto nitrocellulose following separation on a 15% SDS-PAGE gel. Nitrocellulose filters were incubated overnight at 4°C with 1.5 μg/ml of $p16^{INK4a}$ monoclonal antibody. Detection was performed using enhanced chemical luminescence as recommended by the manufacturer (ECL Amersham, Arlington Heights, IL).

3. Results

Since AK lesions consisting of transformed keratinocytes often progress into SCC, we investigated p53 and $p16^{INK4a/ARF}$, the most commonly altered genes in SCC, for the integrity of their loci on chromosomes 17p13.1 and 9p21, respectively, in SCC and AK skin samples. Two microsatellite markers were used for each of the analyzed regions. Thirty-eight SCC and 15 AK samples isolated from tumor and the case-matched normal tissues were analyzed for LOH. LOH analysis was performed using an ABI automated DNA sequencer and evaluated with the Genescan software. An example demonstrating LOH at D9S162 in a SCC (case number 15) is shown in Fig. 1.

Ninety-seven percent of SCCs (37/38) and 93% of AKs (14/15) were heterozygote and informative for at least one of the loci on 17p13.1 (Tables 1 and 2). LOH in the 17p13.1 region was detected in 54.5% of SCC when analyzed by the D17S938 microsatellite marker and in 64.5% when D17S786 was tested (Table 1). In AK samples, these numbers were 50 and 45%, respectively (Table 2).

LOH in at least one locus of the 17p13.1 region was detected in 84% of SCCs and in 64% of AKs, indicating that in both SCCs and AKs alterations in the region encoding the p53 tumor suppressor gene are similarly frequent (Table 3).

Also 97% of SCCs (37/38) and 93% of AKs (14/15) were informative for at least one loci in the 9p21 region (Tables 4 and 5). In SCCs LOH of the 9p21 microsatellite markers was seen in 37% for D9S162 and 33% for D9S171 (Table 4). LOH in at least one locus of the 9p21 region was observed in 46% of SCC (Table 6). In AK samples the numbers were 20 and 10%, respectively (Tables 5 and 6).

These results illustrate the similarities between SCC and AK in the p53 status and confirm the previous findings emphasizing the role of p53 and $p16^{INK4a}$ in the development of SCC [2,4,16,17]. More importantly, however, these data indicate the differences in the $p16^{INK4a}$ status between AK and SCC lesions as judged by the fact that among 64% of the AK lesions with altered p53 locus only 21% had some alterations in the $p16^{INK4a}$ locus. The 21% is most likely a great overestimation due to AK heterogeneity discussed below. Nevertheless, LOH in the

Table 3
Distribution analysis of LOH in the p53 locus

	LOH in D17S938	LOH in D17S786	At least one LOH
SCC (%)	54.5 (18/33)	64.5 (20/31)	84 (31/37)
AK (%)	50 (5/10)	45 (5/11)	64 (9/14)

Table 4
Summary of microsatellite instability and LOH in the 9p21 region in 38 patients with SCC

Marker	Case number[a]																																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
D9S162	●	⊗	●	⊗	●	⊗	●	⊗	⊗	⊗	●	○	⊗	⊗	●	●	◒	⊗	●	○	⊗	⊗	⊗	⊗	●	●	⊗	⊗	⊗	◒	●	⊗	⊗	●	○	⊗	⊗	●
D9S171	●	○	⊗	⊗	⊗	○	●	⊗	○	⊗	⊗	⊗	⊗	○	●	○	◒	⊗	●	○	⊗	⊗	○	●	⊗	○	●	⊗	●	●	○	⊗	○	⊗	⊗	⊗	○	●

[a] ●, LOH; ◒, microsatellite instability; ○, homozygous; ⊗, heterozygous and retention cases.

Table 5
Summary of microsatellite instability and LOH in the 9p21 region in 15 patients with AK

Marker	Case number[a]														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D9S162	○	⊗	⊗	◒	○	⊗	●	⊗	○	⊗	○	●	⊗	○	⊗
D9S171	⊗	○	⊗	⊗	○	⊗	○	⊗	⊗	⊗	⊗	⊗	⊗	●	○

[a] ●, LOH; ◒, microsatellite instability; ○, homozygous; ⊗, heterozygous and retention cases.

$p16^{INK4a/ARF}$ locus appears to be less frequent in AK lesions than in SCC.

To determine whether the genomic aberrations in the 9p21 region result in the abrogation of the $p16^{INK4a}$ protein expression we performed immunohistochemistry with monoclonal antibodies detecting $p16^{INK4a}$ antigen in ten SCC and 12 AK tissue sections (Fig. 2A). While 66% of AK samples were positively stained with the $p16^{INK4a}$ antibody, only 10% of SCC samples showed positive staining (8/12 vs. 1/10). The $P < 0.05$ value of the χ^2-test indicates that the differences at the protein level are statistically significant. This was confirmed by the Western blot (Fig. 2B) of normal, AK and SCC cell lysates, showing equal expression of $p16^{INK4a}$ in normal and AK cells and its absence in the SCC cells.

All together these results indicate that most SCC but not AK cells are defective in $p16^{INK4a}$ expression and suggest that progression of AK to SCC involves inactivation of the $p16^{INK4a/ARF}$ tumor suppressor.

4. Discussion

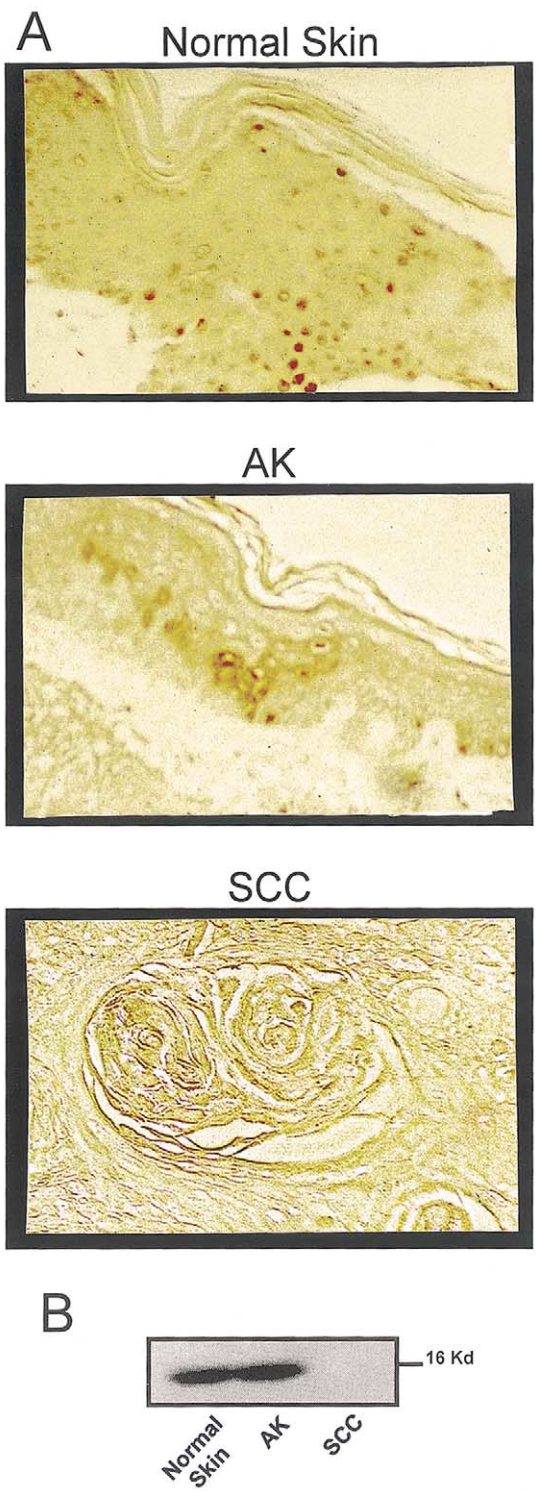
The lower than p53 incidence of the $p16^{INK4a}$ inactivation in SCC, but their high correlation, imply that the genetic alterations of the p53 gene or inactivation of its product are the early events in SCC development followed by the $p16^{INK4a}$ abnormalities. Here, we examined the integrity of the $p16^{INK4a/ARF}$ locus in AK and SCC cells by LOH analysis and correlated it with $p16^{INK4a}$ expression at the protein level detected by Western blot and immunohistochemistry. The $p16^{INK4a}$ appears to be less frequently inactivated in AK lesions than in SCCs.

Table 6
Distribution analysis of LOH in the p16 locus

	LOH in D9S162	LOH in D9S171	At least one LOH
SCC (%)	37 (13/35)	33 (9/27)	46 (17/37)
AK (%)	20 (2/10)	10 (1/11)	21.4 (3/14)

AKs are prevalent and seen in elderly patients with fair skin and a history of excessive sun exposure. UVR in sunlight appears to be a major factor responsible for the development of AKs as evidenced by the presence of UV-specific mutations in lesional keratinocytes with a transformed phenotype. Neoplastic keratinocytes of AK lesions are confined to the epidermis but they invade the dermis at a rate of 10% per year for an individual lesion with a variation of 0.1–16% and disease progresses to invasive SCC [18,19]. Given that each patient has multiple AK lesions, the risk of developing SCC increases significantly for the AK patients. Recently it has been suggested that AK is in fact a SCC at its early stage of development [3]. This conclusion is based on the observations that AKs frequently progress to SCCs, and that the cytological atypia of AK cells and cells of SCC inside the dermis or in metastatic lesions are identical, although AKs and SCCs differ in their clinical appearance and extension of lesions. Moreover, molecular studies determined that p53, the most commonly mutated tumor suppressor gene in SCCs, is also a common site of mutational alterations in the AK keratinocytes [20]. Furthermore, in both cases the introduced mutations are of the UV type, strongly implying the solar irradiation as a common initiation factor [4]. Consequently, Cockerell [3] proposes a classification of KA as a first stage, the KIN I (keratinocytic interepidermal neoplasia) of evolving SCC analogous to CIN I (cervical intraepithelial neoplasia) of evolving carcinoma of the uterine cervix.

The molecular events responsible for the progression of AK lesions in situ to invasive SCCs remain unknown. In this study we accumulated data implying



that this progression requires inactivation of the $p16^{INK4a}$ function in addition to the alterations in p53. The $p16^{INK4a/ARF}$ locus in the 9p21 region encodes two alternatively spliced mRNAs translated into $p16^{INK4a}$ (exons 1α, 2 and 3) and $p14^{ARF}$ (exons 1β, 2 and 3) proteins [30]. These two proteins are involved in regulation of the cell cycle progression. Indeed, $p14^{ARF}$ is able to bind and inhibit MDM2 and prevent p53 degradation, otherwise imposed by MDM2 [21,22]. Consequently, $p14^{ARF}$ is able to act both at the G1/S phase and the G2/M phase and inhibit cell proliferation in a p53-dependent manner. $p16^{INK4a}$ arrests cells in the G1 phase of the cell cycle in a pRB-dependent fashion [23,24] by redistribution of CDK4 from cyclin D to $p16^{INK4a}$ protein to form a $p16^{INK4a}$–CDK4 complex. Unbound cyclin D is rapidly destroyed by the ubiquitin-dependant proteasome pathway [25] and this stops the cell cycle progression.

We found that KA keratinocytes express $p16^{INK4a}$ at the level comparable with the normal cells, while SCC cells exhibit a decreased expression of this protein, implying that uninhibited SCC cell growth is a consequence of the $p16^{INK4a}$ inactivation rather than $p14^{ARF}$. Recent publications support this conclusion. Indeed, $p16^{INK4a}$ knockout mice developed squamous carcinoma after UVR [26] and the overexpression of wild-type $p16^{INK4a}$ inhibited growth of murine SCC [27]. Moreover, Soufir et al. [16] showed that the mutation of the $p16^{INK4a}$ gene in SCC is of the UV type.

Our data incorporated into the multistep skin carcinogenesis model [28] imply inactivation of the $p16^{INK4a}$ function as a crucial molecular event leading to progression of AK to SCC of the skin. Perhaps a clonal expansion of surviving cells with altered p53 is recognized clinically as AK. AK lesions contain transformed keratinocytes, but as we found most of them still express normal levels of the $p16^{INK4a}$. As apoptosis-resistant, however, these cells continue to accumulate UV-induced mutations, including those inactivating $p16^{INK4a}$.

Fig. 2. Representative samples showing immunohistochemistry (A) and Western blot (B) with anti-$p16^{INK4a}$ antibody. Immunohistochemistry (magnification 400×) and Western blot of 100 μg of protein lysates prepared from normal keratinocytes, AK and SCC cells was performed as described in Section 2. Staining with actin antibody served as a control (data not shown).

We propose that inactivation of $p16^{INK4a}$ advances AK cells to the next step in development of SCC. Histopathology of AK lesions and immunohistochemistry with anti-$p16^{INK4a}$ antibody provide additional evidence supporting this notion. Indeed, $p16^{INK4a}$ positive cells are not seen in the highly displastic areas within the AK lesions. Perhaps keratinocytes in these displastic, $p16^{INK4a}$ negative areas already advanced to SCC. The mixture of $p16^{INK4a}$ positive and negative cells within the same AK lesions points to their heterogeneous character. This most likely is a reason for the statistically insignificant differences between AK and SCC skin samples when the LOH analysis was used, but is highly significant in the immunohistochemistry studies despite the lower number of analyzed samples. The $p16^{INK4a}$ staining distinguishes the already $p16^{INK4a}$ negative SCC areas from those still $p16^{INK4}$ positive AK regions within the same AK lesions. The LOH analysis at the DNA level apparently is less sensitive making this distinction more difficult. To confirm this, LOH analysis of AK tissue samples at different stages of development in conjunction with dissection of displastic regions to separate them from the still histologically normal tissue will be needed. This may help to determine the stage at which AK acquires $p16^{INK4a}$ alterations and to correlate to the KIN grade of Cockerell [3]. The heterologous $p16^{INK4a}$ staining also offers a diagnostic test to detect AK that is already on the way to SCC. A disruption of the basal membrane which distinguishes AK from SCC involves even more genetic abnormalities, including perhaps a downregulation of retinoic acid receptors found recently to correlate with the progression of AK to SCC [29].

In conclusion, our data provide strong evidence that progression from AK to SCC involves molecular changes leading to $p16^{INK4a}$ inactivation. This evidence supports Cockerell's hypothesis [3] that AK and SCC are the same disease but at different stages of development.

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