In an attempt to investigate the neoplastic progression in different stages of actinic keratosis (AK), a standardized AgNOR analysis was performed in 94 cases of AK, 35 of which were associated with squamous cell carcinoma (SCC) or basal cell carcinoma (BCC), and in 31 cases of SCC and 22 cases of BCC. The cases were subdivided into low- and high-AgnOR-expressing (AgNOR status) AK by using the mean area of AgNORs per cell (NORA) value (3.996 µm²) as the cut-off. In AK samples, a progressive increase of the mean NORA value from Stage I to Stage IV was encountered. In addition, a significantly higher mean NORA value was found in the AK cases associated with SCC, in comparison to those without SCC; by contrast, no significant differences in the mean NORA value were noted between AK cases with or without BCC. A highly significant association between a high AgNOR quantity and the coexistence of SCC was encountered in AK; no association was appreciable between the AgNOR quantity and the co-occurrence of BCC. Moreover, when the co-existence of SCC in AK was considered as the reference point, the AK cases associated with SCC mostly (95.5%) presented a high AgNOR quantity (high sensitivity), but only 57.6% of cases without SCC displayed a low AgNOR quantity (low specificity). Additionally, our data document that the standardised AgNOR analysis represents a strong negative predictor for the association between SCC and AK. Indeed, a low AgNOR quantity mostly is associated with AK cases without SCC.

Key words: Actinic keratosis, squamous cell carcinoma, AgNORs, standardized AgNOR analysis, proliferation.
as of skin tumors (Egan et al. 1988; Rüschoff et al. 1990; Delahunt et al. 1991; Eusebi et al. 1991; De Rosa et al. 1992; Pich et al. 1992; Aubele et al. 1994; Pich et al. 1994; Öfner et al. 1995b; Antonangelo et al. 1997; Giuffrè et al. 1998; Tuccari et al. 1999; Giuffrè et al. 2001; Giuffrè et al. 2006). Moreover, in a previous study (Tuccari et al. 2001), we applied for the first time the standardized procedure proposed by the European Committee on AgNOR Quantification (Öfner et al. 1995b) to determine the AgNOR quantity in the different stages of actinic keratosis, thus identifying those cases with proliferative characteristics and a strong propensity to develop infiltrative SCC (Tuccari et al. 2001); we concluded that in AK a progressively increased cell cycle speed proceeded in the meantime the epithelial dysplasia appears and it is extended to the different layers of the epidermis (Tuccari et al. 2001). Moreover, the highest mean AgNOR quantity encountered in AK Stage IV was very similar to that found in SCC, suggesting a similar proliferation rate in these two skin lesions (Tuccari et al. 2001). We analysed herein the AgNOR quantity in cases with co-existing AK and SCC or BCC in comparison to data coming from AK alone in order to assess the predictive value of AgNOR method in the identification of aggressive and evolutive AK.

Materials and Methods

One hundred-forty seven sun-exposed skin surgical samples were collected from files of our Institution, including 94 cases of AK (53 male, 41 female; mean age 67.9 years, ranging from 37 to 88 years), 35 of which were associated with co-existing SCC (22) or BCC (Giuffrè et al. 1998). All cases were graded according to criteria of Hashimoto and Mehregan (Hashimoto et al. 1990) in four histological stages: 23 Stage I (Basal layer atypia), 28 Stage II (Lower epidermal dysplasia), 23 Stage III (Middle epidermal dysplasia) and 20 Stage IV (Total epidermal dysplasia). Moreover, 31 cases of SCC (14 male, 17 female; mean age 71.4 years, ranging from 58 to 93 years), 22 of which were AK-associated, and 22 cases of BCC (17 male, 5 female; mean age 67.5 years, ranging from 53 to 83 years), 13 of which were AK-associated, were also analyzed. Finally, 10 normal skin fragments were taken during laparotomic surgery and utilized as tissue control. The assessment of diagnosis and staging was obtained by 2 pathologists with a good correlation and reproducibility; in the cases evaluated in different manner, consensus was reached after discussion using a double-headed microscope.

All surgical samples had been fixed in 10% neutral formalin for 12-24 hrs at room temperature and then embedded in paraffin at 56°C. From each tissue block, two consecutive 4 µm thick sections were cut and mounted on silane-coated glasses, then dewaxed in xylene, rehydrated in graded ethanols and submitted to haematoxylin and eosin (H&E) stain and to the AgNOR technique according to guidelines of the Committee on AgNOR Quantification (Öfner et al., 1995a), respectively. In detail, sections were immersed in sodium citrate buffer (pH 6) and incubated in wet autoclave at 120°C (1.1-1.2 bar, at sea level) for 20 min and then allowed to cool down to 37°C. Subsequently, slides were immersed in a freshly prepared silver-staining solution containing one part of volume of 2% gelatin in 1% formic acid and two parts of 25% aqueous silver nitrate solution, at 37°C in a thermostatically controlled environment for 11 min. The reaction was then stopped by washing the slides with bidistilled-deionized water in order to remove unwanted silver precipitates. Finally, all sections were dehydrated in ascending ethanols, clarified in xylene and mounted with a synthetic medium (Permount, Fisher Scientific, Hampton, New Hampshire, USA).

The quantification of AgNORs was performed by an image analysis system consisting of an optical microscope (DM RB, Leica Microsystems) fitted with a single chip color CCD video camera (Ikegami ICD-840PDC, Ikegami Tsushinki Co. Ltd., Tokyo, Japan) having a resolution of 460x420 (horizontal x vertical) TV lines and an image processing unit installed in a computer. For each slide examined, microscopic fields representative of the lesions were assessed excluding areas in which regressive changes as well as technical artifacts were present, as compared with the corresponding H&E stained section. The mean area (µm²) of AgNORs per cell (NORA) was evaluated using cells in one focal plane with a x40 objective lens in at least 100 nuclei per specimens (mean 130); specific softwares, IM 5200 (Microscience Inc., Phoenix Technology Inc., Seattle, Washington, USA) and AgNOR (Immagini e Computer, Rho-Milan, Italy).
were utilized to determine mean NORA values per cell and per case, respectively.

After testing the normal distribution of NORA values in all groups of patients by Kolmogorov-Smirnov test, parametric tests were applied. A statistical descriptive analysis (mean value and standard deviation) was performed for each case of different groups of cutaneous lesions, while differences among categories were assessed by analysis of variance and the Newman-Keuls’ multiple comparisons test. Moreover, the AK cases were dichotomously divided into low- and high-AgNOR-expressing (AgNOR status) by using the mean NORA value encountered in AK as the cut-off point. Finally in AK, differences between AgNOR status and co-existence or not with cutaneous neoplasms (SCC or BCC) were assessed by Fisher’s exact test. Sensitivity, specificity, negative predictive value, positive predictive value of AgNOR status were also evaluated taking as reference point the co-existence of SCC or BCC. A probability (p) value less than 0.05 was considered statistically significant. Data were analyzed using the SPSS package version 6.1.3 (SPSS Inc., Chicago, IL, USA).

Results

An adequate silver-staining intensity, homogeneously present throughout the whole section, was appreciable in all the AK, as well as in the neoplastic and control cutaneous fragments by AgNOR method. Mature lymphocytes, whenever present, exhibited a single round-shaped, centrally localized AgNOR.

In AK lesions, the AgNORs were clearly distinguishable as black dots in the nuclei of the epidermal basal layer as well as within the nucleoli (Figure 1 a, b); moreover, irregularly shaped aggregates of AgNORs were present in disarrayed large hyperchromatic pleomorphic as well as in dyskeratotic cells, which occupied half the thickness of the epidermis and may reach the granular layer. In AK samples, the mean NORA value was 3.996 µm² (SD ±1.014) with a progressive increase in the mean NORA value (Figure 1 a, b) moving from Stage I to Stage IV (Table 1). Highly significant differences (p<0.001) were noted when Stages I or II were compared with Stages III or IV, respectively; in addition, a significantly higher mean NORA value (p<0.001) was obtained in the Stage III cases in

Figure 1. A moderate amount of intranuclear silver precipitates in a case of AK considered as low-AgNOR-expressing (a); a larger and diffuse quantity of intranuclear black dots in a high-AgNOR-expressing AK case (b) (Standardized AgNOR method, scale bar represents 20 µm).
comparison to those with a Stage IV. By contrast, no significant difference was found between Stages II and I of AK cases.

In 59 AK cases without any evidence of SCC or BCC, the mean NORA value was 3.786 µm² (SD ±0.955). Conversely, 22 cases of AK associated with SCC showed a mean NORA value of 4.969 µm² (SD±0.507), while a lower mean NORA value was encountered in 13 AK co-existing with BCC (3.303 µm²; SD±0.796). A highly significant difference (p<0.001) was found in the comparison of AK cases with or without co-existence of SCC; no differences were noted between AK cases with or without the presence of BCC.

In SCC, the neoplastic elements showed an evident increase of AgNORs in the nucleus, mostly organized in irregularly shaped aggregates (Figure 2); the number and distribution of the intranuclear black dots were strikingly different from that of normal skin. The mean NORA value was 5.374 µm² (SD±0.666) and it was very similar to that encountered in AK Stage IV (5.247 µm²; SD±0.554). In detail, cases of pure SCC showed a mean NORA value of 5.193 µm² (SD±0.924), while SCC associated to AK cases exhibited a mean NORA value of 5.448 µm² (SD±0.536). No statistical differences between these two latter groups of SCC were noted.

In BCC, the AgNORs were mainly localized in the basal elements with a scattered pattern (Figure 3); the corresponding NORA value was 3.092 µm² (SD ±0.323). In detail, cases of pure BCC showed a mean NORA value of 3.050 µm² (SD±0.251), while BCC associated to AK cases exhibited a mean NORA value of 3.126 µm² (SD±0.380). No statistical differences between these two latter groups of BCC were noted.

In the control normal skin specimens, the lowest

<table>
<thead>
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<th>No.</th>
<th>NORA</th>
<th>SD</th>
<th>Significance</th>
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<tr>
<td></td>
<td>NORA</td>
<td>SD</td>
<td>p-value</td>
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<tr>
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<td>±0.104</td>
</tr>
<tr>
<td>Stage I</td>
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<td>±0.762</td>
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<tr>
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<td>23</td>
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<tr>
<td>Stage IV</td>
<td>20</td>
<td>5.247</td>
<td>±0.554</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
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</tr>
<tr>
<td>Basal cell carcinoma</td>
<td>22</td>
<td>3.092</td>
<td>±0.323</td>
</tr>
<tr>
<td>Normal skin</td>
<td>10</td>
<td>1.869</td>
<td>±0.332</td>
</tr>
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mean NORA value was found (1.869 µm²; SD ±0.332) (Figure 4).

When all specimens were subdivided into four groups, namely normal skin, BCC, AK and SCC, a highly significant difference in the mean NORA value (p<0.001) was found among them, although some degree of overlap among single mean NORA values was encountered.

Regarding the AgNOR status, AK cases were subdivided into 35 low- and 46 high-AgNOR-expressing (Figure 1 a, b), taking into consideration the value of 3.996 µm² as the cut-off point. A highly significant association between AgNOR status and coexistence of SCC (p<0.00001) was encountered in AK (Table 2); no association was appreciable between AgNOR status and co-existence of BCC. Moreover when the AK cases were stratified by stage, among 40 AK cases of I-II stage, 32 cases without any association with SCC were low-AgNOR-expressing but 8 cases were high-AgNOR-expressing, 7 of which were SCC-associated (p<0.00001). By contrast, in AK cases of III-IV stage, 38/41 were high-AgNOR-expressing and 14/41 were SCC-associated; only 3 cases were low-AgNOR-expressing, 2 of which were not SCC-associated (p=0.70122).

Finally, taking as a reference point the co-existence of SCC in AK, the probability that AK cases associated with SCC display a high AgNOR quantity (sensitivity) was 95.5% (21/22 AK with SCC) whereas the probability that a case without SCC was characterized by a low AgNOR quantity (specificity) was 57.6% (a low AgNOR quantity was detected in 34/59 cases of AK without SCC). The probability that a highly AgNOR expressing AK was associated with SCC (positive predictive value) was 45.7% (21/46 highly AgNOR expressing AK cases were associated with SCC), while the probability that a low AgNOR expression identified a case of AK without SCC (negative predictive value) was 97.1% (34/35 cases with a low AgNOR quantity were not associated with SCC).

Discussion

In order to differentiate AK from malignant epithelial cutaneous tumors, only few studies using the AgNOR method, exclusively based on a subjective counting procedure of intranuclear silver dots, have been carried out (Egan et al. 1988; Heinisch et al. 1994; Onodera et al. 1996; Khanna et al. 2001; Romão-Corrêa et al. 2005); in some reports, the AgNOR numbers in AK were significantly lower than those observed in SCC as well as in BCC (Egan et al. 1988; Onodera et al. 1996). Nevertheless, a comparative AgNOR analysis among different AK stages has been already investigated by our group by using the standardized AgNOR method (Tuccari et al. 2001). This previous study was the first in which the standardized AgNOR analysis had been performed in cutaneous preneoplastic and neoplastic pathology according to the guidelines of the Committee on AgNOR Quantification (Öfner et al. 1995a). In particular, by wet-autoclave pre-treatment, we obtained a constantly high staining quality of single-interphase AgNORs, irrespective of the duration of formalin fixation and archival storage, similarly to that elsewhere reported (Öfner et al. 1996). Moreover, the quantification of AgNOR area was made by an image analyzer system, being more objective, free of observer bias and less time consuming than counting AgNORs by eye (Rüschoff et al. 1990b; Öfner et al. 1996). Therefore, the evaluation of changes in the number and shape of nucleoli present in malignant cells from human skin cancers, performed by not standardized recommended procedures (Romão-Corrêa et al. 2005), should be considered with caution; in fact, if the AgNOR evaluation in skin tumours is approached by subjective counts of single or aggregate specific silver precipitates, either intranuclear or intranucleolar, conflicting data about BCC and SCC have been reported (Egan et al. 1988; Khanna et al. 2001).

In the present series, utilizing the above mentioned standardized procedure, we have found a highly significant difference of the AgNOR quantity among AK, BCC and SCC, examined as pure enti-
ties, thus suggesting a progressive increase of the proliferation rate moving from preneoplastic lesions to malignant neoplasias. Moreover, in AK cases, the AgNOR analysis showed a progressive increase of the mean NORA values moving from Stage I to Stage IV, with a highly significant difference among low stages and Stage III or IV; by contrast, no difference was evident between Stage I and II. Furthermore, when we compared AK cases with or without co-existence of SCC, a highly significant difference ($p<0.001$) emerged while no differences were appreciable between AK cases with or without the presence of BCC. On the other hand, pure SCC cases exhibited a quantity of AgNORs quite similar to that encountered in AK cases of Stage IV, whereas lower mean NORA values were found in pure BCC. No significant differences were achieved when a comparison was made between pure SCC or AK-associated SCC as well as between pure BCC or AK-associated BCC. Of course, the lowest mean NORA value was present in the control normal skin specimens. Finally, considering the mean NORA value of AK cases as a cut-off point in order to define low- and high-AgNOR-expressing cases, a highly significant association between AgNOR status and coexistence of SCC ($p<0.00001$) was encountered; by contrast, no association was appreciable between AgNOR status and co-existence of BCC. However, taking as reference point the co-existence of SCC in AK, the sensitivity of AgNOR status was very high (95.5%), with a down-specificity; additionally, our data document the standardised AgNOR analysis represents a strong negative predictor (negative predictive value =97.1%) for the association between SCC and AK. Moreover, this aspect appears greatly evident when only low stage AK cases were considered; in fact, all cases displaying a low AgNOR quantity showed no association with SCC, while 7/8 characterized by a high AgNOR quantity were SCC-associated. In biological terms, it is well established the AgNOR quantity allows to obtain information on the rate of ribosome biogenesis in pathological tissue specimens (Derenzini et al., 2000); in fact, the AgNOR distribution is strictly related to the RNA polymerase I activity, so an higher AgNOR value corresponds to a greater rRNA transcription (Derenzini et al., 2000). However, an up-regulation of ribosome biogenesis enhancing the cellular proliferation rate has been reported in breast cancer, in association with an altered status in pRB and p53 (Trerè et al., 2004); therefore, the nucleolar hypertrophy and the high ribosome biogenesis may represent the final effects of the loss of the inhibitory effects on pRB and p53 on RNA polymerase I activity (Trerè et al., 2004). On the light of these considerations, in AK a significantly increased AgNOR quantity may characterize those cases with deleted pRB and mutated p53, identifying patients with deregulated cell cycle and able to easily progress towards SCC.

Therefore, these latter findings further improve our understanding of pathobiological changes accompanying the progression of AK into SCC. On the basis of our observations, the determination of AgNOR quantity may represent an useful additional tool to identify patients, mainly with low stage of AK, able to develop a co-existent SCC. However, from a practical point of view in dermatopathology, the AgNOR may be utilized as an objective and inexpensive method in the assessment of the rank of risk in AK, mainly when skin lesions are incompletely removed by small biopsies or partial excisions.

References


