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Comparative histochemical study of Bowen's disease and actinic keratosis: preserved normal basal cells in Bowen's disease

H. Ishida¹, M. Kumakiri¹, K. Ueda¹, L.M. Lao¹, M. Yanagihara², K. Asamoto³, Y. Imamura⁴, S. Noriki⁴, and M. Fukuda⁴

Departments of ¹Dermatology, ³Anatomy and ⁴Pathology, Fukui Medical University, Matsuoka, Yoshida-Gun, 910-1193 Fukui, Japan and Departments of ²Dermatology, Kanazawa Medical University, Uchinada, Kahoku-Gun, 920-0293 Ishikawa, Japan

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SUMMARY

The degree of DNA-instability as revealed by immunohistochemical staining with anti-cytidine antibody after acid hydrolysis (DNA-instability test) has been recently used as a marker of malignancy. This technique was applied to examine 17 skin tissue samples of Bowen's disease, 47 of actinic keratosis, 15 of squamous cell carcinoma, 5 of seborrheic keratosis, and 10 of normal skin. All benign neoplastic cells of seborrheic keratosis and normal epidermal cells were negative. On the other hand, all cancer cells were positive with the DNA-instability test, indicating their malignancy, but all basal cells in Bowen's disease were completely negative. Compatible with this result, the basal cells in Bowen's disease were characteristically normal as evident in other histochemical examinations. Thus, they were negative with p53 immunohistochemistry, with normal signals of chromosome 17 *in situ* hybridisation and argyrophilic nucleolar organiser region, and showed slightly enhanced proliferative activity as revealed by proliferating cell nuclear antigen immunohistochemistry. Immunohistochemical staining with 34 β E12 (monoclonal antibody against cytokeratins

1, 5, 10, and 14), which stains all normal epidermal keratinocytes including basal cells, showed that only the basal cells of Bowen's disease stained strongly and homogeneously, while all cancer cells in the upper layers of Bowen's disease and all layers of actinic keratosis were only sporadically or weakly stained. Staining with 34 β B4 (monoclonal antibody against cytokeratin 1), which recognises the whole epidermis except for the basal layer in the normal epidermis, showed that the basal cells in the Bowen's disease were completely negative, and lower layer cells in the actinic keratosis and upper layer cells in Bowen's disease were only sporadically stained positive, although the superficial layer cells in actinic keratosis stained strongly and homogeneously. Our findings clearly indicate that the basal cells in Bowen's disease are normal. In support of this conclusion, the same cells showed normal morphology on electron microscopy with preserved basement membrane, although the latter was often damaged in actinic keratosis.

Correspondence to: M. Fukuda
E-mail: west@fmsrsa.fukui-med.ac.jp

INTRODUCTION

Both Bowen's disease and actinic keratosis are intraepidermal squamous cell carcinomas with potential progression to invasive squamous cell carcinoma (Sober and Burstein, 1995; Kirkham, 1997; Mackie, 1998). The percentage of cases with Bowen's disease and actinic keratosis that progress to invasive squamous cell carcinoma are 4 (Thestrup-Pedersen *et al.*, 1988) to 5% (Graham and Helwig, 1973), and 8% (Glogau, 2000), respectively.

Bowen's disease occurs mainly in sun-exposed skin areas (Thestrup-Pedersen *et al.*, 1988; Kossard and Rosen, 1992) and is possibly caused by ultraviolet light (UV) (Reizner *et al.*, 1994; Chuang *et al.*, 1995), or on the lower extremities (Cox, 1994). A number of studies have demonstrated a relatively high incidence of human papilloma virus (HPV) in Bowen's disease affecting the genital region (38-80%) and other skin regions (19.4-40%), by using Southern blot analysis with HPV 16 DNA (Ikenberg *et al.*, 1983), immunohistochemical staining with viral antigens (Grussendorf-Conen and Giesen, 1990), and *in situ* hybridisation (ISH) (Kettler *et al.*, 1990). Bowen's disease is also an important complication of chronic hyperarsenism (Yeh *et al.*, 1968; Miki *et al.*, 1982). Histologically, highly atypical cancer cells with clumping lie in complete disorder throughout the epidermis (Kirkham, 1997).

On the other hand, actinic keratosis manifests as hyperkeratotic lesions in sun-exposed adult skin (Mackie, 1998), and the incidence of the disease has been increasing lately (Suzuki *et al.*, 1996). Five types of actinic keratosis have been described; hypertrophic, atrophic, bowenoid, acantholytic, and pigmented, based on histopathological examination. Transition and combination of these types are also known to occur (Kirkham, 1997). Histologically, the intraepidermal units of eccrine ducts and hair canals are spared from the cancer lesion (Hashimoto and Mehregan, 1990), and normal skin grows over the lesion at the borders forming the slanted lines of demarcation (Pinkus, 1958). Bowen's disease and bowenoid type actinic keratosis are histologically identical, except for the accompanying actinic elastosis in the latter, but they are indistinguishable when the former occurs on the sun-exposed skin areas (Hashimoto and Mehregan, 1990; Kirkham, 1997).

The basal layer in Bowen's disease may, however, appear free of dysplasia, in contrast to that of

actinic keratosis (Hashimoto and Mehregan, 1990; Lee and Wick, 1990). Basal and suprabasal cells in Bowen's disease also appear almost normal on electron microscopy, except that the tonofilaments in these cells slightly aggregate in a fascicular form (Seiji and Mizuno, 1969). Immunohistochemically, the basal cells are either weakly positive or completely negative for the expression of proliferating cell nuclear antigen (PCNA) (Geary and Cooper, 1992; Takata and Matsui, 1994; Li and Lee, 1996), although the PCNA index (percentage of PCNA-positive cells) is generally high in Bowen's disease (Tsuji *et al.*, 1992).

In the present study, we investigated whether the basal cells in Bowen's disease were normal, and determined the differences in biological characteristics between Bowen's disease and actinic keratosis.

Several histochemical and immunohistochemical techniques have been so far applied to skin cancers. The number of AgNOR (argyrophilic nucleolar organiser region)-signals/nucleus is higher in malignant tumours than in their benign counterparts (Crocker and Skilbeck, 1987; Mackie *et al.*, 1989; Yu *et al.*, 1992). Kanitakis *et al.* (1992) reported the presence of significantly higher AgNOR count in squamous cell carcinoma (SCC) compared with keratoacanthoma. Onodera *et al.* (1996) found the highest mean number of AgNOR in SCC lesions, followed in order by Bowen's disease, basal cell carcinoma, actinic keratosis, and seborrheic keratosis. Using interphase cytogenetics, the mean number of signals of chromosome 1 and 17 was significantly higher in malignant melanomas than in other melanocytic neoplasms (De Wit *et al.*, 1994; Matsuta *et al.*, 1994). Hatchoh *et al.* (1992) demonstrated numerical abnormalities of chromosome 7 in SCC. By using flow cytometry, high aneuploidy rates were detected in both Bowen's disease (90-95%) and actinic keratosis (40-69%) (Newton *et al.*, 1987; Biesterfeld *et al.*, 1995). P53 immunoreactivity was observed in malignant skin tumours including Bowen's disease and actinic keratosis, but not in seborrheic keratosis and benign melanocytic tumours (McGregor *et al.*, 1992; Ro *et al.*, 1993). Furthermore, UV-specific double-base changes and mutations at dipyrimidine sites were found in the p53 gene of SCC (Brash *et al.*, 1991; Ziegler *et al.*, 1994), and overexpression of p53 protein was demonstrated in Bowen's disease induced by chronic arsenic exposure (Kuo *et al.*, 1997; Hsu *et al.*, 1999).

Thus, the above methods have demonstrated abnormal gene expression, protein synthesis, disturbed cytodifferentiation, and stimulated proliferative activity of cancer cells reflecting biological changes. However, these methods cannot be used as consistent and specific markers for distinguishing cancer cells from normal cells because the abnormalities found by these methods were only statistically significant. On the other hand, Fukuda *et al.* (1986, 1993) indicated that nuclear DNA of cancer cells is without exceptions always more unstable compared to that of normal and benign tumour cells, irrespective of epithelial or mesenchymal origin. They also developed the method of differential fluorescent or immunohistochemical staining of cancer cells after denaturation of DNA by acid hydrolysis.

In the present study, we used this feature of DNA-instability of cancer cells to identify normal cells remaining in the basal layer of Bowen's disease, together with other histochemical and immunohistochemical methods to characterise the biological characteristics of cancer cells.

MATERIALS AND METHODS

Immunohistochemical staining of paraffin-embedded sections with polyclonal anti-single-stranded DNA antibody after acid hydrolysis to demonstrate DNA-instability (DNA-instability test)

(A) Materials

Skin biopsies or surgically resected skin specimens were obtained from 17 patients with Bowen's disease, 47 patients with actinic keratosis (hypertrophic type, 17; atrophic type, 18; acantholytic type, 10; bowenoid type, 2), 15 patients with squamous cell carcinoma, 5 patients with seborrheic keratosis, and 10 subjects with normal skin. The specimens were fixed in 10% buffered formalin for 24 hours at room temperature. Sections were then embedded in paraffin then cut into 4- μ m thick sections, deparaffinized with xylene and replaced by ethanol.

(B) Preblocking

After washing in water, intrinsic peroxidase was blocked with 0.3% H₂O₂ solution dissolved in absolute methanol at 20°C for 15 minutes and

rinsed with PBS at pH 7.4. Sections were treated with 2% skim-milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 20 min to block the background adsorption of antiserum.

(C) HCl hydrolysis and immunohistochemical staining of single-stranded DNA by the ABC method

Sections were then washed in water, and acid hydrolysis with 2N HCl at 30°C was performed for 20 min. Sections were washed in water, again then reacted with the blocking solution (Histofine, Nichirei Corp., Tokyo, Japan). They were reacted with polyclonal antibody to cytidine (Biogenesis, UK, 1:1500 dilution) at 4°C overnight and rinsed with PBS (pH 7.4). Negative controls were provided by omission of the primary antibodies. Sections were reacted with biotinylated goat anti-rabbit IgG (Histofine, Nichirei) at 37°C for 40 min, and rinsed with PBS (pH 7.4) and allowed to react with the avidin-biotin peroxidase complex (Histofine, Nichirei) at 37°C for 30 min and then rinsed with PBS pH 7.4.

(D) Co-DAB reaction

In order to visualise the peroxidase colour reaction, sections were incubated with DAB (3,3'-diaminobenzidine tetrahydrochloride)-cobalt solution (DAB, Lot. CK114, Dogin, Kumamoto, Japan, 50 mg dissolved in 100 ml of 0.05 M Tris-HCl buffer pH 7.4, added with 2 ml of 1% CoCl₂ and 10 μ l of 30% H₂O₂ solution) at 20°C for 10 min and washed in water. Nuclear counterstaining was carried out with Kernechtrot (Kernechtrot, Chroma, Stuttgart, Germany, 0.1 g and 5 g aluminium sulphate dissolved in 100 ml distilled water). The slides were dehydrated through graded alcohol solutions followed by xylene, and mounted.

Immunohistochemical staining for PCNA, p53, and cytokeratins

(A) Staining methods

Serial 4 μ m thick sections were prepared from the same specimens used for the DNA-instability test as described above. After washing with water, intrinsic peroxidase was blocked with 0.3% H₂O₂ solution dissolved in absolute methanol at 20°C for 15 min and washed with PBS (pH 7.4). They were digested with 0.1% trypsin solution (pH 7.2) (Sigma, St. Louis, MO, USA) at 37°C for 15 min only for cytokeratin. All sections including those for

cytokeratins were irradiated twice in a poly-propylene slide holder with a cap filled with PBS (pH 7.4), over a period of 5 min, using a microwave oven (500 W, ER-245, Toshiba, Tokyo). Sections were then treated with 2% skim-milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 20 min to block the background adsorption of the antiserum. They were further reacted with the blocking solution (LSAB Kit, Lot.00046, Dako, Carpinteria, CA, USA) at 20°C for 5 min. This was followed by reaction with the following primary antibodies, respectively, at 4°C overnight: monoclonal anti-PCNA antiserum (PC-10, NC-012, Lot. 511, Novocastra Laboratories, Newcastle, UK, 1:50 dilution), monoclonal anti-p53 antiserum (DO-1, Lot. 48920216, Oncogene Research Products, USA, 1:40 dilution), monoclonal anti-cytokeratin antiserum (34 β E12, Lot. 044C, Dako, Carpinteria, CA, USA, 1:40 dilution), monoclonal anti-cytokeratin antiserum (34 β B4, Lot 10GDA6, Enzo Diagnostics, NY, USA, 1:40 dilution). Negative controls were provided by omission of the primary antibodies. Then the sections were washed with PBS (pH 7.4), and further reacted with biotinylated anti-mouse IgG (LSAB Kit) at 37°C for 30 min, followed by rinsing with PBS (pH 7.4). They were allowed to react with the avidin-biotin-peroxidase complex (LSAB Kit) at 37°C for 30 min and rinsed with PBS (pH 7.4). In order to visualise the peroxidase colour reaction, section were incubated with DAB solution (20 mg dissolved in 100 ml of 0.05 M Tris-HCl buffer [pH 7.4]) at 20°C for 10 min and washed in water. Nuclear counterstaining was performed with hematoxylin. The slides were dehydrated by an alcohol series followed by xylene, and mounted.

(B) Evaluation of immunoreactivity

As per p53 staining, tissue samples containing > 10% of positively stained cells were regarded as positive. The extent of PCNA-positivity was evaluated by determining the positively stained nuclei in at least 500 epithelial cells in each specimen using an Olympus microscope ($\times 40$ objective and $\times 10$ eye piece).

AgNOR

(A) Staining method

Specimens taken from the same tissues used in other experiments were used for AgNOR staining.

Deparaffinized and alcohol treated specimens were washed in de-ionised and distilled water, and coated with 2% gelatin dissolved in 0.001% formic acid solution added with twice the volume of 50% silver nitrate, and allowed to react at 20°C for 20 min under a safety light (No. LA. Kodak, NY, USA). The specimens were then fixed with Fuji-fix solution (Fujifix, Fuji Photo Film, Tokyo, Japan, 445 g dissolved in 2,000 ml of de-ionised and distilled water) for 1 min at room temperature and thoroughly washed in de-ionised and distilled water. No nuclear counterstaining was performed. The slides were dehydrated through an alcohol series followed by xylene, and mounted.

(B) Quantitative analysis of the number, largest size, and maximum shape irregularity of AgNOR

The colour image analyser MacScope version 2.56 (Mitani Corporation, Japan) was used for analysis. The image of an optical field of a specimen stained by the AgNOR technique was made by using the objective lens ($\times 100$). The total number of AgNOR contained in one optical field or in each nucleus was counted manually in the image shown on the cathode ray tube (CRT). Three to five optical fields, namely about 100 cells, were examined in each specimen. The mean value of the maximum shape irregularity of AgNOR expressed as the degree of deviation from a regular circle was determined automatically for the most irregularly-shaped AgNOR in 10 nuclei for each specimen, by the same analyser, on the photograph printed from the image viewed on the CRT with a final magnification of $\times 1,000$.

In situ hybridisation

(A) Processing of paraffin sections

4 μ m paraffin sections were cut from the same tissues used in other experiments and floated in warm (40°C) water followed by mounting on silanised slides (DAKO). The slides were air dried overnight at 40°C, dewaxed in xylene and rehydrated through a decreasing alcohol series to water. The slides were then immersed in methanol supplemented with 0.3% H₂O₂ for 15 min to block endogenous peroxidase activity. After washing with distilled water and rinsing in PBS (pH 7.4), they were digested with 0.25% trypsin solution (Sigma) at 37°C for 20 to 60 min. The slides were

finally washed with PBS twice for 5 min, rinsed in distilled water, and processed for ISH.

(B) In situ hybridisation

The probe used in this study was a biotin labelled α -satellite DNA probe specific for the pericentromeric region of chromosome 17 (D17Z1, Lot. 5C219, Oncor, Gaithersburg, MD, USA). A hybridisation mixture was prepared which consisted of 50% formamide, 20% dextran sulphate, $2 \times$ SSC (0.3 M NaCl, 0.03 M sodium citrate and 0.04 M disodium hydrogen phosphate, pH 6.0), 10 μ g/ml carrier salmon testes DNA (Lot. M9E4924, Nacalai tesque, Kyoto, Japan), 1/50 Denhardt's solution, and biotin-labelled probe (10 μ l/ml of the hybridisation solution). The mixture was denatured at 73°C for 10 min and rapidly cooled on ice. The slides were denatured by immersion in a solution containing 70% formamide in $2 \times$ SSC at 73°C for 10 min and rapidly cooled by rinsing in 70% cold ethanol (prechilled at -20°C for 5 min), followed by sequential dehydration in 70%, 80%, 90%, and 100% ethanol at room temperature. The slides were air dried and incubated with the hybridisation mixture, and covered with a cover slip. Hybridisation was carried out at 37°C in a moist chamber containing 50% formamide and $2 \times$ SSC for 48 hours. Following hybridisation, the slides were immersed in $2 \times$ SSC to remove the cover slips. Post hybridisation washing was done in 50% formamide in $2 \times$ SSC at 41°C for 10 min, 30% formamide in $2 \times$ SSC at 37°C for 8 min, and $1 \times$ SSC at room temperature for 5 min. The slides were then rinsed in distilled water and dehydrated through increasing concentrations of ethanol. The bound probe was visualised by air drying the slides at room temperature followed by the conventional ABC method of immunodetection using DAB as chromogen and H₂O₂ as substrate. The slides were counterstained with hematoxylin, dehydrated through an alcohol series followed by xylene and were finally mounted.

(C) Scoring of interphase nuclei

After hybridisation, at least 200 nuclei were counted from each section using a conventional light microscope with an oil immersion lens ($\times 100$). In all specimens, signal in stromal cells and lymphocytes served as an internal control. In each section, counts of cells showing nil, 1, 2, 3, 4, 5, or more hybridised signals were determined and the

percentage of hyperdiploid cells (3 or more signals within 1 nucleus) was determined in each specimen.

Electron microscopy

For routine electron microscopic observation, tissues were fixed in 2.5% glutaraldehyde buffered with PBS (pH 7.4) at 4°C and postfixed in 1% osmium tetroxide in the same buffer for 1 hour. After dehydration in a series of graded concentrations of alcohol, the tissues were embedded in Epon 812. The 1 μ m semi-thin sections were stained with toluidine blue to select appropriate areas for study. Ultrathin sections were cut by an ultratome (Ultracut N, Reichert-Nissei) with a diamond knife, doubly stained with uranyl acetate and lead citrate, and examined and photographed under an electron microscope (H-600, Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV.

Reticulin silver impregnation

4 μ m paraffin sections were cut from the same tissues used in other experiments and dewaxed in xylene and rehydrated through a decreasing alcohol series to water. The slides were immersed in 0.25% potassium permanganate for 10 min. After washing with distilled water, they were immersed in 2% oxalic acid for 2 min, washed with distilled water, then immersed in 2% ferric ammonium sulphate for 50 sec. After washing with water, they were rinsed in three changes of distilled water for 2 min each and immersed for 15 min in a solution prepared by adding in order: 10 ml of 10% silver nitrate, 5 ml of 4% potassium hydroxide, a small quantity of 28% ammonia solution, 185 ml of distilled water. Then they were rinsed briefly in 95% ethyl alcohol and immersed successively in 120 ml of 1% formalin added with 2 ml of 2% ferric ammonium sulphate for 3 min. After washing with distilled water, they were immersed in 1% gold chloride for 10 min or longer. After washing with distilled water, they were immersed in 2% oxalic acid for 20 sec. After washing with water, they were then fixed with Fuji-fix solution (Fujifix, Fuji Photo Film, Tokyo, Japan, 445 g dissolved in 2,000 ml of de-ionised and distilled water) for 1 min, thoroughly washed in distilled water, dehydrated through an alcohol series followed by xylene and were finally mounted.

Statistical Analysis

PCNA-index, AgNOR parameters, and ISH data are expressed as mean \pm standard deviation (SD). Differences between groups were examined for statistical significance using the Student's *t*-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

The results of the DNA-instability test, immunohistochemical staining of PCNA, p53, quantitative analysis of AgNOR-parameters and chromosome 17 ISH are summarised in Tables I and II. The negative controls for all immunohistochemical stainings including the DNA-instability test gave completely negative staining.

DNA-instability test

The DNA-instability test was positive in SCC (Fig. 1a), and negative in seborrheic keratosis (Fig. 1b); virtually all neoplastic cells were positively stained in positive cases and negatively stained in negative cases. All layers in hypertrophic, atrophic, and acantholytic type actinic keratosis, and all layers except for basal layer in bowenoid type actinic keratosis and in Bowen's disease showed positivity indicating increased DNA-instability comparable to that of malignant tumours (Fig. 1c, d). The basal cells in bowenoid type actinic keratosis and in Bowen's disease, and normal epidermal cells were completely negative (Fig. 1d).

PCNA immunohistochemistry

PCNA indices were significantly higher in upper layer cells in Bowen's disease, SCC, and actinic keratosis than those of basal cells in Bowen's disease, normal skin, and seborrheic keratosis. Moreover, PCNA indices of the upper layers in Bowen's disease and in bowenoid type actinic keratosis were statistically higher than those of hypertrophic, atrophic, and acantholytic type actinic keratosis. No difference in PCNA indices were found in the basal layer of Bowen's disease and bowenoid type actinic keratosis, seborrheic keratosis, and normal skin. PCNA labelled cells were mainly recognised at the periphery of the nests in SCC (Fig. 1e), in the lower layers in hypertrophic, atrophic, and acantholytic type actinic keratosis

(Fig. 1f), and in the whole epidermis except for the basal layer in Bowen's disease and in bowenoid type actinic keratosis (Fig. 1g, h).

AgNOR parameters

The values of AgNOR-number were significantly higher in the upper layers in Bowen's disease, actinic keratosis, and SCC (Fig. 1i, j, k) than those in the basal layer in Bowen's disease, seborrheic keratosis and normal skin. In the upper layers of Bowen's disease and bowenoid type actinic keratosis, the values of AgNOR-number were significantly higher than those of hypertrophic, atrophic, and acantholytic type actinic keratosis, but the values of largest size and maximum shape irregularity of AgNOR were significantly lower than those of them (Fig. 1j, k, l). The values of largest size and maximum shape irregularity in SCC were highest. In AgNOR-parameters, no difference was found in the basal layer in Bowen's disease, in bowenoid type actinic keratosis, and in normal skin.

p53 immunohistochemistry

Thirty-four cases of actinic keratosis were positive for p53 immunostaining (Fig. 1m), but no difference was found between hypertrophic, atrophic, and acantholytic type actinic keratosis. Eight cases of Bowen's disease and 1 case of bowenoid type actinic keratosis were positive, but the basal cells of these positive cases were negative (Fig. 1n).

Interphase Cytogenetics

A total of 45 cases from the specimens used in the above experiments were studied by this method. The control normal epidermis showed 0, 1 or 2 signals/nucleus. Similarly, stromal cells and lymphocytes also showed 0, 1 or 2 signals/nucleus. Cells exhibiting 3 or more chromosome copies/nucleus were recognised in all cases of Bowen's disease, actinic keratosis (Fig. 1o, p), and SCC. The percentage of hyperdiploid cells was significantly higher in actinic keratosis and SCC than in Bowen's disease. No difference was found between hypertrophic, atrophic, and acantholytic type actinic keratosis. No hyperdiploid cells were recognised in basal layer cells in Bowen's disease (Fig. 1p) and seborrheic keratosis. ISH methods in two cases of bowenoid type actinic keratosis were unfortunately unsuccessful.

Table I

Results of DNA-instability test, immunohistochemical staining of PCNA and p53, quantitative analysis of AgNOR parameters, and chromosome 17 ISH. Statistical analyses by Student's t-test were performed for the values of PCNA-index, AgNOR parameters, and chromosome 17 ISH using the values for the basal layer of Bowen's disease, seborrheic keratosis, and the basal layer of normal skin. Cases with meaningful differences ($p < 0.05$) are shown by stars. *: mean of hypertrophic, atrophic, and acantholytic type

	Bowen's disease (n=17)		actinic keratosis* (n=45)	squamous cell carcinoma (n=15)	seborrheic keratosis (n=5)	basal layer of normal skin (n=10)
	basal layer	upper layers				
DNA-instability test	all negative	all positive	all positive	all positive	all negative	all negative
PCNA-index(%)	9.2±4.0	60.2±14.2★	51.2±13.5★	55.0±16.6★	13.2±6.4	8.3±2.6
p53 (number of positive cases)	all negative	8 cases (47.1%)	33 cases (73.3%)	6 cases (40.0%)	all negative	all negative
AgNORs number	1.49±0.14	3.22±0.42★	2.26±0.49★	2.84±0.56★	1.53±0.22	1.40±0.14
maximum shape irregularity	0.31±0.10	0.67±0.09	0.77±0.05★	0.82±0.04★	0.61±0.05	0.34±0.07
largest size (µm ²)	2.44±0.90	4.45±1.16	9.79±3.56★	12.7±6.11★	4.20±1.38	2.61±0.85
chromosome 17(%) (% of nuclei with more than 3 ISH spots)	0 (n=10)	8.4±4.6★ (n=10)	13.2±6.9★ (n=23)	19.3±6.7★ (n=7)	0 (n=5)	0 (n=10)

Cytokeratin immunohistochemistry

Immunohistochemical staining with 34 β E12 (monoclonal antibody against cytokeratins 1, 5, 10, and 14), which stains all normal epidermal keratinocytes including basal cells, showed strong and homogeneous staining of the basal cells of Bowen's disease and bowenoid type actinic keratosis. Only sporadic or weak staining was noted in all cancer cells in the upper layers of Bowen's disease and bowenoid type actinic keratosis, and all layers of hypertrophic, atrophic, and acantholytic type actinic keratosis (Fig. 2a, b, c). Immunohistochemical staining with 34 β B4 (monoclonal antibody against cytokeratin 1), which recognises the whole epidermis except for the basal layer in the normal epidermis, showed lack of staining in the basal cells in Bowen's disease and bowenoid type actinic keratosis. Furthermore, only sporadic staining was noted in the lower layer cells in the hypertrophic, atrophic, and acantholytic type actinic keratosis, and upper layer cells in Bowen's disease and bowenoid type actinic keratosis. However, homogeneous and strong staining was noted in the superficial layer cells in hypertrophic, atrophic, and acantholytic type actinic keratosis (Fig. 2d, e, f).

Electron microscopy

The following ultrastructural changes were observed mainly in the spinous layer in cases of Bowen's disease (Fig. 3a): central displacement and aggregation of condensed tonofilaments; decrease in tonofilament-desmosomal attachment; multinucleated giant cells; and abnormal mitosis. The basal cells in Bowen's disease generally exhibited normal appearance, which were characterised by normal polarity, normal cytoplasmic features such as tonofibrils, lipid droplets and mitochondria (Fig. 3a). They were connected to the neighbouring healthy cells or tumour cells by normal desmosomes, and fixed on the basement membrane by half-desmosomes with normal appearance and numbers (Fig. 3b). The well-developed basement membrane sharply separated the epidermis from dermis

Table II

Comparison of different biological parameters (PCNA index, p53, AgNOR parameters, and chromosome 17 ISH) in subtypes of actinic keratosis. Statistical analyses by Student's t-test were performed for the values of PCNA-index and AgNOR parameters using the values for the upper layers of bowenoid type. Cases with meaningful differences ($p < 0.05$) are shown by stars

DNA-instability test	bowenoid type (n=2)		hypertrophic type (n=17)	atrophic type (n=18)	acantholytic type (n=10)	mean of hypertrophic atrophic, and acantholytic type, (n=45)	
	basal layer	upper layers				all positive	all positive
PCNA-index(%)	all negative	all positive	48.8±12.4★	51.6±13.6★	54.4±15.5	51.2±13.5★	
p53 (number of positive cases)	all negative	1 cases (50.0%)	13 cases (76.5%)	13 cases (72.2%)	7 cases (70.0%)	33 cases (73.3%)	
AgNORs number	1.30±0.08	3.13±0.45	2.34±0.58	2.21±0.51★	2.22±0.27★	2.26±0.49★	
maximum shape irregularity	0.33±0.02	0.72±0.02	0.76±0.06	0.76±0.05	0.81±0.03★	0.77±0.05	
largest size(µm ²)	1.71±0.07	5.85±2.47	10.8±5.11	8.58±2.53	10.5±1.63★	9.79±3.56	
chromosome 17(% of nuclei with more than 3 ISH spots)	n. d.	n. d.	15.8±7.09 (n=8)	11.2±7.76 (n=10)	13.2±3.70 (n=5)	13.2±6.9 (n=23)	

and was intact in all examined tissue specimens (Fig. 3b).

At the dermal-epidermal junction of the lesions in hypertrophic, atrophic, and acantholytic type actinic keratosis, the basement membrane showed sporadic discontinuity and a partial decrease in the number of half-desmosomes, in addition to focal separation from the basal cells (Fig. 3c). The presence of double or multiple basement membranes was often observed. The desmosomes between tumour cells seemed to be normal.

Reticulin silver impregnation

In Bowen's disease, positively-stained fibres appeared to be continuous without any disruptions at the dermal-epidermal junction (Fig. 4a), but those in hypertrophic, atrophic, and acantholytic type actinic keratosis were often partially disrupted (Fig. 4b).

DISCUSSION

Fukuda *et al.* (1986, 1992) reported that DNA-instability is a specific and common marker of malignancy irrespective of epithelial or mesenchymal origin. Based on this finding, they developed the method of differential staining of malignant cells with acridine orange or anti-single-stranded DNA antiserum based on the phenomenon of increased production of denatured single-stranded DNA in cancer cells than in comparable normal cells after acid hydrolysis. Based on the degree of DNA-instability by acid hydrolysis, as revealed in the present study, our results reconfirmed that seborrheic keratosis belongs to the benign category, while SCC, actinic keratosis, and Bowen's disease were found to belong to the malignancy category.

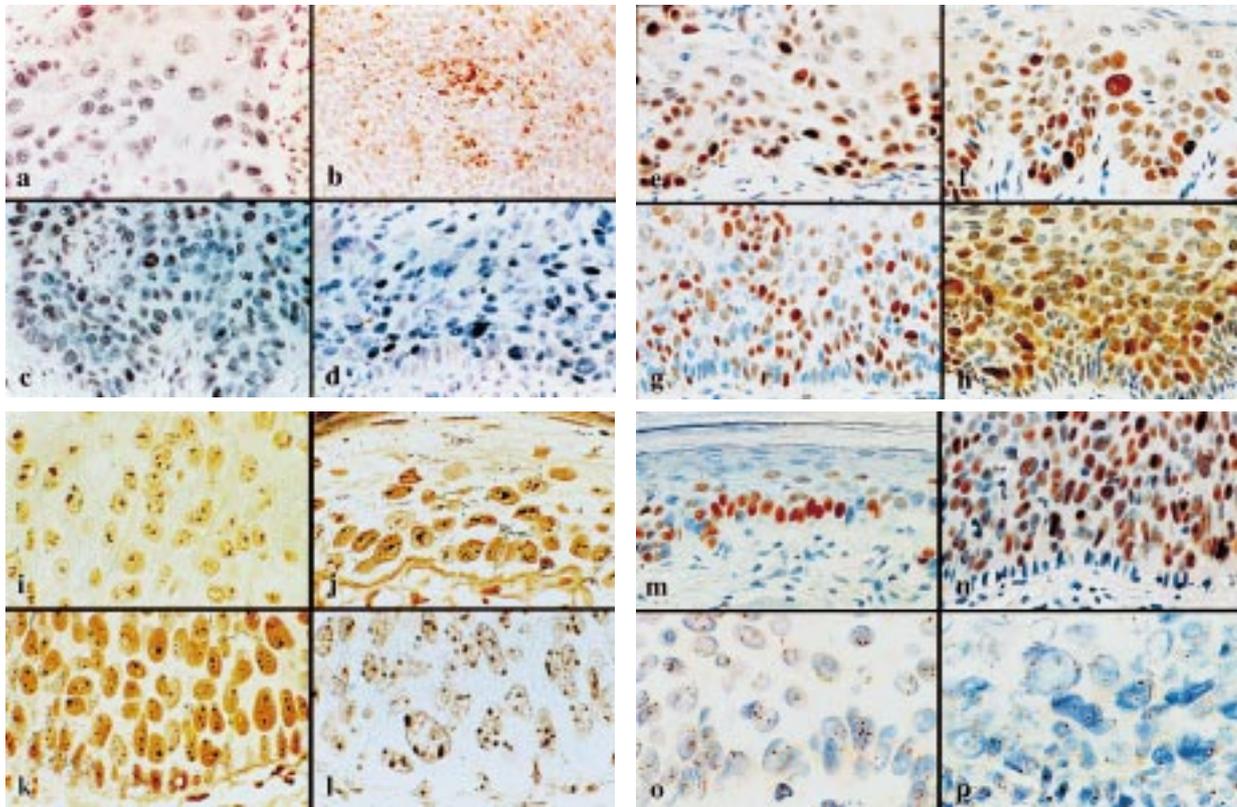


Fig. 1 - Immunohistochemical staining of anti-single stranded DNA antibody (a-d). a: SCC (×132), b: seborrheic keratosis (×132), c: actinic keratosis (×132), d: Bowen's disease (×132). Immunohistochemical staining of PCNA (e-h). e: SCC (×100), f: actinic keratosis (×132), g: Bowen's disease (×132), h: bowenoid type actinic keratosis (×132). AgNORs staining (i-l). i: SCC (×250), j: actinic keratosis (×250), k: Bowen's disease (×250), l: bowenoid type actinic keratosis (×330). Immunohistochemical staining of p53 (m-n). m: actinic keratosis (×132), n: Bowen's disease (×132). Chromosome 17 ISH signals (o-p). o: actinic keratosis (×250), p: Bowen's disease (×330).

Cytokeratins are the major structural proteins of epithelial cells. Monoclonal antibody 34 β E12 (against cytokeratins 1, 5, 10, and 14) stains all normal epidermal keratinocytes including basal cells (Gown and Vogel, 1982) and squamous cell

carcinoma (Gown and Vogel, 1985). On the other hand, monoclonal antibody 34 β B4 (against cytokeratins 1) recognises the whole epidermis except for the basal layer in the normal epidermis (Gown and Vogel, 1984). Immunohistochemical

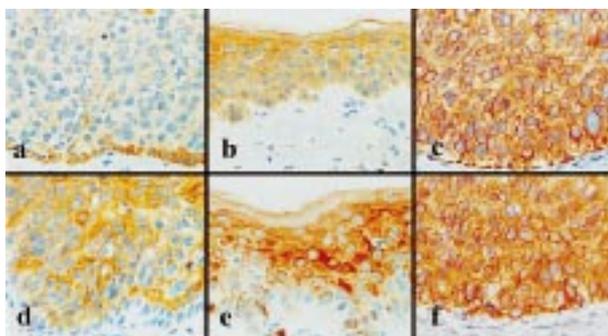


Fig. 2 - Immunohistochemical staining of cytokeratin (34 β E12) (×132) (a-c). a: Bowen's disease, b: actinic keratosis, c: bowenoid type actinic keratosis. Immunohistochemical staining of cytokeratin (34 β B4) (×132) (d-f). d: Bowen's disease, e: actinic keratosis, f: bowenoid type actinic keratosis.

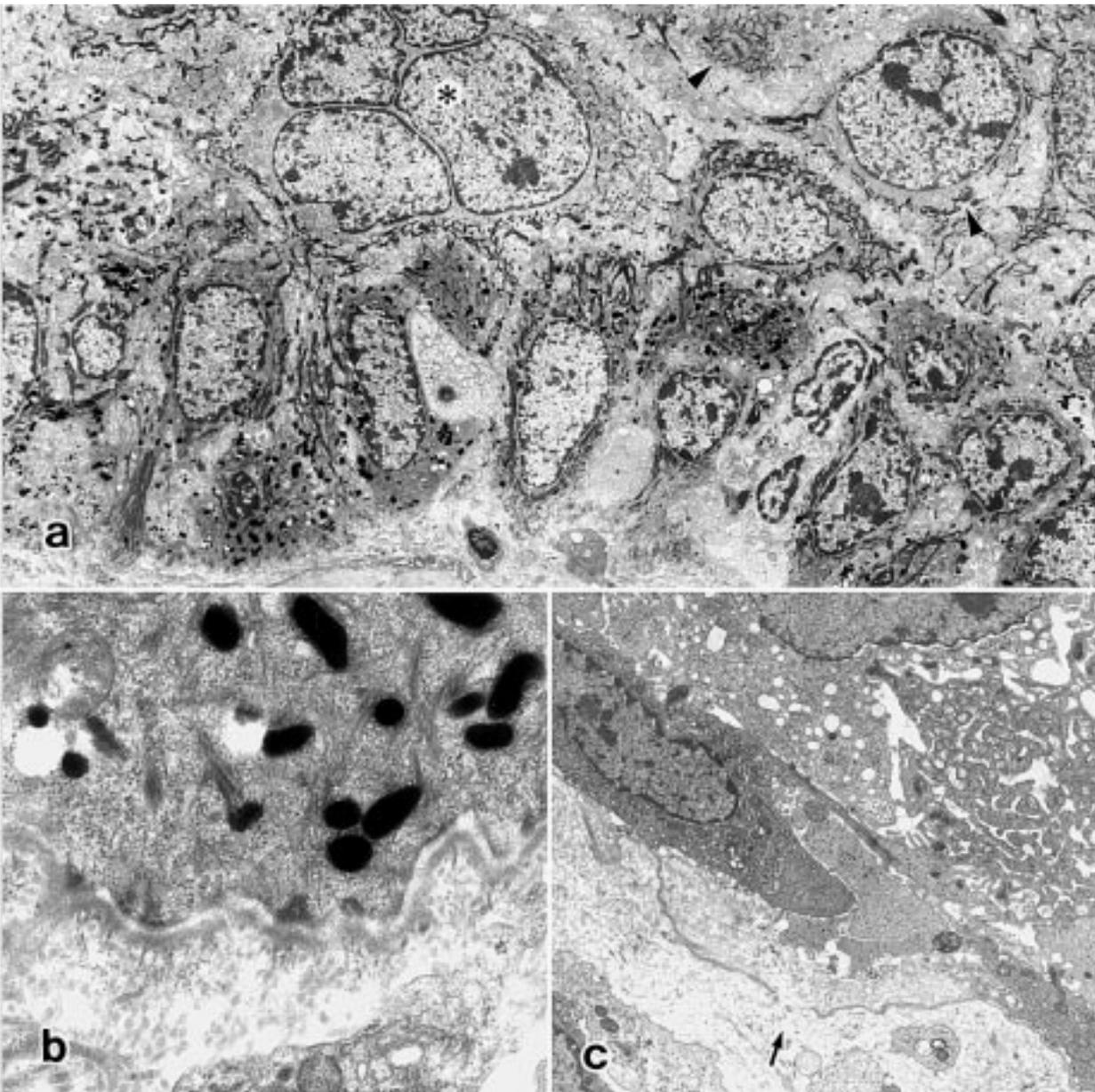


Fig. 3 - Electron microscopy (a-c). **a:** Electron micrograph of the lower part of the epidermis from a patient with Bowen's disease. Note the multinucleated giant cells (*) and dyskeratotic cells (arrowheads) in the spinous layer. ($\times 2,200$), **b:** High magnification of a portion of the basal cell from A. ($\times 25,000$), **c:** Basal layer from the lesion of actinic keratosis. The arrow indicates a focal separation of basement membrane from the basal cells. ($\times 6,000$).

staining of tissue samples of Bowen's disease and actinic keratosis in the present study using these antibodies showed a heterogeneous staining pattern in tumour cells except for the basal layer in Bowen's disease and in bowenoid type actinic keratosis. Specifically, with 34 β E12, these cancer cells showed only sporadic or weak staining. With

34 β B4, the lower layer cells in the hypertrophic, atrophic, and acantholytic type actinic keratosis, and upper layer cells in Bowen's disease and in bowenoid type actinic keratosis showed sporadic staining. However, the superficial layer cells in hypertrophic, atrophic, and acantholytic type actinic keratosis were strongly and homogeneous-

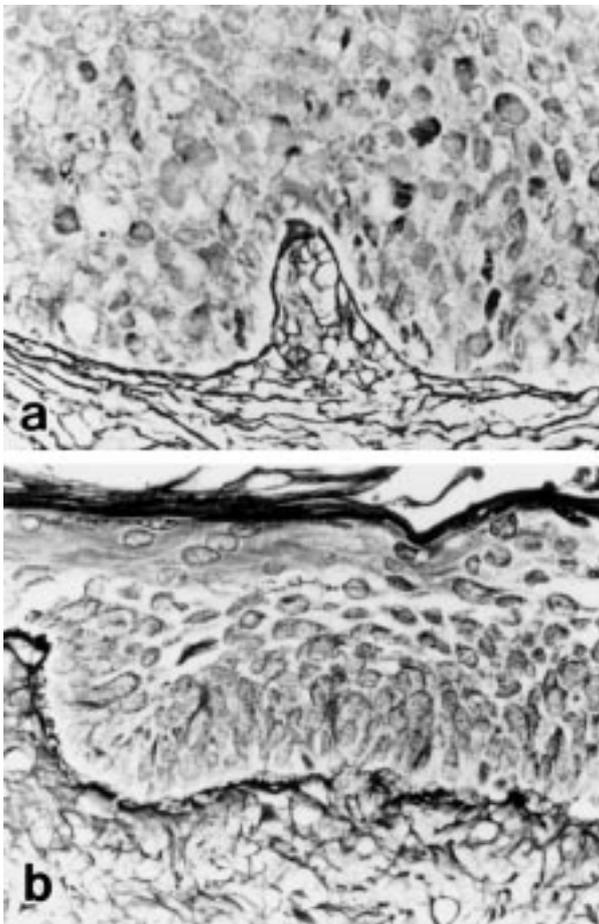


Fig. 4 - Reticulin silver impregnation ($\times 132$) (a-b). **a:** Bowen's disease, **b:** actinic keratosis.

ly stained with 34 β B4. Only the basal cells of Bowen's disease and bowenoid type actinic keratosis showed a staining pattern that was similar to that of the basal cells of normal epidermis; they were strongly and homogeneously stained with 34 β E12 and were completely negative with 34 β B4.

The basal layer of Bowen's disease may appear free of dysplasia, in contrast to that of actinic keratosis, on light microscopy (Hashimoto and Mehregan, 1990; Lee and Wick, 1990) and electron microscopy (Seiji and Mizuno, 1969). It is, however, not always easy to characterise this layer as benign based upon simple routinely used morphological criteria, because cellular atypia in malignant tumours is not usually markedly pronounced. The correlation between morphological appearance and biological characteristics may not always be satisfactory and reliable. In the DNA-instability test, all

basal cells in Bowen's disease were completely negative. Compatible with this result, the characteristics of basal cells in Bowen's disease were comparable to those of normal basal cells examined by other histochemical techniques; they were negative with p53 immunohistochemistry, showed normal signals of chromosome 17 in ISH and AgNOR, revealed slightly enhanced proliferative activity by PCNA immunohistochemistry, and showed similar staining pattern to the normal basal cells by cytokeratin immunohistochemistry.

All these observations clearly indicate that the basal cells in Bowen's disease are biologically normal. In support of this conclusion, the basement membrane in Bowen's disease appeared normal, in contrast to that in actinic keratosis, which showed sporadic discontinuities by electron microscopy and reticulin silver impregnation stain. It is reasonable to speculate that the presence of a normal basal cell layer is related to the notable infrequency with which Bowen's disease progresses to invasive carcinoma (Graham and Helwig, 1973; Thestrup-Pedersen *et al.*, 1988) and to the frequent recognition of large macules or plaques in Bowen's disease, regardless of the presence of markedly atypical epidermal cells and high growth fraction. On the other hand, atypical cells were distributed both in the basal and suprabasal layers by microscopic and histochemical examinations in hypertrophic, atrophic, and acantholytic type actinic keratosis. Both Bowen's disease and actinic keratosis are thought to arise from basal cells with proliferative activity. In Bowen's disease, however, cancer cells cannot remain in the basal layer for one reason or another and presumably they proliferate first in the spinous layer to take the form of intraepidermal carcinoma, and continue proliferating further to replace normal epidermal cells, leaving only basal cells. The underlying mechanism of such a proliferating pattern in Bowen's disease remains unknown. However, it is speculated that Bowen's disease undergoes changes different from those of actinic keratosis with respect to cell adhesion in the early stage of carcinogenesis, thus making it impossible for cancer cells to remain in the basal layer in the former. Alternatively, it is possible that the proteins that constitute half-desmosomes necessary for adhesion with the basement membrane are abnormal in Bowen's disease. The appearance of intercellular desmosomes was

almost normal on electron microscopy in Bowen's disease. Previous studies have shown that the expression of E-cadherin, one of the constituent proteins in intercellular adherens junction, does not differ from that of the normal epidermis (Fuller *et al.*, 1996), although a low expression level of desmoglein 1, one of the constituent proteins of desmosomes, has been reported (Tada *et al.*, 2000). However, there are no reports describing the presence of abnormal constituent proteins of half-desmosomes. Half-desmosomes in Bowen's disease are the subject of future studies, and Bowen's disease will serve as an interesting material for studying intercellular adhesion.

Actinic keratosis appears to show a tendency to normal differentiation as revealed by cytokeratin staining. On the other hand, Bowen's disease shows an intense nuclear atypia and no tendency for differentiation. Our results revealed that the PCNA-index, a marker of proliferative activity, was higher in Bowen's disease than in actinic keratosis. In actinic keratosis, however, the frequency of abnormality of chromosome 17 was high and mutation of p53 was also noted at high frequency compared with those in Bowen's disease. These findings suggest that actinic keratosis and Bowen's disease differ with respect to carcinogenesis, and that the DNA damage may be more prominent in actinic keratosis than in Bowen's disease. The signals of AgNOR in Bowen's disease, except for the normal basal cells, were small and round although the number was large, while those in actinic keratosis were larger and more irregularly shaped although the number was smaller, like those in invasive squamous cell carcinoma. These results clearly indicate that the malignant potential of actinic keratosis is higher than that of Bowen's disease irrespective of the degree of morphological atypism. Previous studies have suggested that progression of actinic keratosis to invasive squamous cell carcinoma is rather rare (Kirkham, 1997; Mackie, 1998), although more recent studies have reported the incidence to be as high as 8% (Glogau, 2000).

The staining patterns of bowenoid type actinic keratosis were similar to those of Bowen's disease, indicating that the basal cells of bowenoid type actinic keratosis remained normal. These results suggest that bowenoid type actinic keratosis and Bowen's disease are not biologically different. Therefore, it is possible that bowenoid type actinic

keratosis is Bowen's disease that has developed in the part exposed to light.

In conclusion, we have demonstrated in the present study that the basal cells were normal in Bowen's disease and bowenoid type actinic keratosis while those in other types of actinic keratosis were replaced by cancer cells.

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