

Expression of Cell-cycle Proteins p53, p21 (WAF-1), PCNA and Ki-67 in Benign, Premalignant and Malignant Skin Lesions with Implicated HPV Involvement

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A series of 120 biopsies from benign (verruca vulgaris and keratoacanthoma), premalignant (actinic keratosis and extragenital Bowen's disease) and malignant (squamous cell carcinoma) skin lesions were studied immunohistochemically for the expression of cell-cycle proteins p53, p21 (WAF-1), PCNA and Ki-67. The presence of human papillomavirus (HPV) DNA in these samples had been analysed previously using *in situ* hybridization (ISH) and PCR. Moderate to intense expression of both PCNA and Ki-67 was present in most of the lesions studied. PCNA staining was extensive in the epidermis underneath the layers where abundant HPV DNA staining was shown in HPV DNA-positive verrucas. In keratoacanthomas, p21 and PCNA expression remained low, despite intense p53 expression. In actinic keratosis, only half of the specimens showed overexpression of p53 associated with moderate or intense expression of PCNA. In extragenital Bowen's lesions, all these cell-cycle markers were overexpressed, but in squamous cell carcinomas, they were heterogeneously expressed and showed no correlation with tumour differentiation. Our results suggest a mechanism by which HPV can reactivate the host genes (leading to cell proliferation) to support its own DNA replication. Also p21 might start keratinocyte differentiation in areas where HPV DNA replication starts. Cell proliferation remained active in actinic keratosis and Bowen's lesions, emphasizing the precancer character of these lesions in contrast with the benign nature of keratoacanthoma and verruca vulgaris. **Key words:** cell-cycle proteins; human papillomavirus; verruca; keratoacanthoma; actinic keratosis; extragenital Bowen's disease; squamous cell carcinoma.

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Neoplastic diseases, as proliferative disorders, are characterized by uncoordinated cell growth. Activation of proto-oncogenes and inactivation of tumour suppressor genes are the main adverse genetic and epigenetic events that are responsible for neoplastic transformation (1). Of these, the p53 tumour-suppressor gene is the most striking example, found to be mutated in about half of the cancers arising from a wide variety of human tissues (2, 3).

The p53 protein was discovered in 1979 as cellular 53-kD nuclear phosphoprotein bound to the large transforming (T) antigen of SV40 virus (4). The effects of wild type p53 (wt p53) is important in the growth control of the cell through arresting the cell cycle in G1, or inducing apoptosis following DNA damage. Accordingly, the loss of normal p53 function

or mutant forms of p53 are associated with cell proliferation and neoplastic transformation (5–7). Normal p53 function can be lost by several mechanisms, e.g. genetic changes (mutations, deletions, structural rearrangement and genomic insertions); formation of protein complexes with viral oncoproteins (SV40 T antigen, high-risk human papillomavirus E6, Epstein-Barr virus nuclear antigen-5 and Epstein-Barr virus immediate-early protein); and binding to cellular gene products (Mdm2) (8, 9).

The p21 protein exists in a quaternary complex with a cyclin, a cyclin-dependent protein kinase (CDK) and proliferating cell nuclear antigen (PCNA). The p21 protein has been suggested to mediate p53-induced growth arrest triggered by DNA damage (10, 11). The p21 protein blocks the initiation of DNA-replication by inhibiting CDK complexes (12, 13). It may also block the action of PCNA (14). The p21 protein has been shown to switch off p53 activation to decreased cell proliferation (15).

As a cofactor for DNA polymerase delta and DNA replication, PCNA expression is a useful marker of cell proliferation in both normal and neoplastic tissues (16, 17). PCNA is induced during the cell-cycle transition from the G0 to G1 phase and further increased during the S phase (18–20). Yet another protein, Ki-67 (a high molecular weight non-histone protein) is present in the G1, S, G2 and M-phases of the cell-cycle, but absent in the G0 cells (21, 22). Several recent studies have shown a good correlation between Ki-67 immunoreactivity and different indices of cell proliferation (23).

Skin carcinogenesis is a multistep process characterized by a series of genetic and epigenetic events resulting in the emergence of cells escaped from the normal growth control mechanisms. Radiation (UV, ionizing), genodermatoses and HPV infection are implicated causes of skin cancer (24–26). Not unlike at other squamous epithelial sites, invasive skin cancer develops through well-defined precancer lesions, i.e. different grades of dysplasia (intraepithelial neoplasia) (26). Thus, the progress of skin carcinogenesis is amenable to close monitoring, by assessing the biological processes throughout the whole spectrum of these precancer lesions. The present study was designed to elucidate the role of the cell-cycle proteins p53, p21, PCNA, Ki-67 and their association with the presence of HPV DNA in benign, premalignant and malignant proliferative skin lesions.

MATERIALS AND METHODS

Specimens

A total of 120 paraffin-embedded blocks of skin lesions were selected from the files of a clinical pathology laboratory (Laboratory of Pathology, Finnish Cancer Society, Kuopio, Finland). All specimens

were derived from the years 1991–94. The specimens included 24 cases of each the 5 distinct skin diseases as follows: verruca vulgaris (VV), keratoacanthoma (KA), actinic keratosis (AK), extragenital Bowen's disease (EBD) and squamous cell carcinoma (SCC). The SCCs were graded into well ($n=4$), moderately ($n=13$) and poorly ($n=7$) differentiated, according to generally accepted criteria. The presence of HPV DNA was analysed in all lesions as reported separately (27–30), using *in situ* hybridization (ISH) (31) and polymerase chain reaction (PCR) (32). HPV DNA was detected only in verruca lesions. All other lesions were HPV DNA-negative in these analyses.

Immunohistochemistry (IHC)

Antibodies. Four different monoclonal antibodies were used in this study: p53 D07, WAF-1 (Novocastra Laboratories Ltd, Claremont Place, UK); PCNA PC10 (DAKO A/S, Glostrup, Denmark) and Ki-67 (Immunotech, Marseille, France). p53 D07 reacts with both the wild-type and the mutant p53.

The avidin-biotin-peroxidase complex immunostaining method was used in this study. The monoclonal antibodies were applied to the sections with their respective dilutions at 4°C, overnight. For providing minimal loss of antigenicity and reducing the risk of false-negative staining, special pretreatments of the sections were used for detection of p53, Ki-67 and p21 proteins with the microwave oven technique, as detailed in Table I. Negative controls were processed in parallel by omitting the primary antibody. Normal skin biopsies were also studied for the expression of PCNA, Ki-67 and p53. The tonsil, breast cancer and verruca vulgaris were used as positive controls for PCNA, p53, Ki-67 and p21, respectively.

Grading of immunostaining. For assessing the immunostaining, a semiquantitative approach was used, based on the proportion of positively stained cells, as follows. Grade 0–1 (weak staining), no or few (<10%) positive cells; grade 2 (moderate staining), 10–50% of positive cells; and grade 3 (strong staining), >50% of positive cells. Furthermore, the IHC-staining pattern was classified as follows: a) basal layer expression; b) basal and suprabasal layer expression (for p21 protein, only suprabasal cells were positive); and c) whole thickness expression. The staining intensity and the staining patterns were scored by 2 independent observers (S.L. and S.S.).

Statistical analysis

In the basic statistical calculations, Chi-square test and Spearman analysis were used with the Statistica 4.5 for Windows software (StatSoft Inc.). The significances were interpreted according to the values in general use, i.e. $p < 0.05$ is significant.

Table I. Pretreatment, dilution and positive controls used for PCNA, p53, Ki-67 and WAF-1

Antibody	Pretreatment	Dilution	Positive controls
PCNA PC10	–	1:100	Tonsil
NCL-p53 DO7	Heating in microwave oven for 5 min	1:100	Breast cancer
Ki-67 MIB-1	Heating in microwave oven for 3 × 5 min with citric acid and citric sodium	1:200	Verruca vulgaris
NCL-WAF-1	Heating in microwave oven for 2 × 5 min with citric sodium	1:10	Verruca vulgaris

RESULTS

The IHC-staining of the cell-cycle proteins p21, p53, PCNA and Ki-67 in the lesions studied is demonstrated in Figs. 1–6.

Verruca vulgaris

The staining of p53 protein was weak and scattered. Moderate to strong immunoreactivity for PCNA and p21 was found in 62.5% and 66.7%, respectively (Figs. 1b, c). In all HPV DNA-positive VV lesions, PCNA expression was extensively present in the epidermis, including the basal, parabasal and spinous cell layers. Intense PCNA signals were

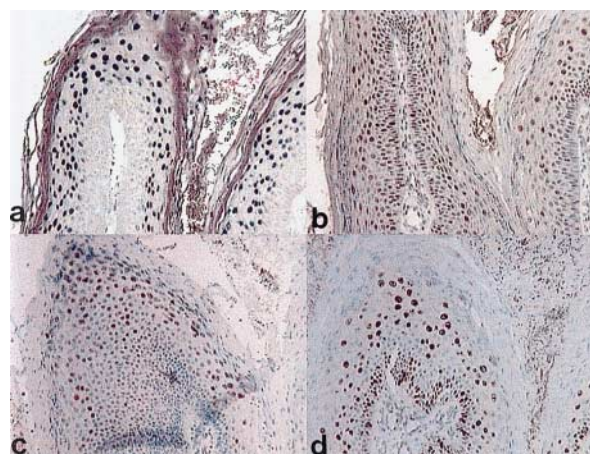


Fig. 1. Verruca vulgaris showing (a) intense HPV DNA type 2 positive signals with *in situ* hybridization. (b) PCNA protein expression is intense and signals are detected below the area where HPV DNA is seen. (c) p21 expression colocalized in the same region as HPV DNA. (d) Ki-67 intensely expressed and the staining pattern is partly different from that found with PCNA. (Original magnification 100 ×).

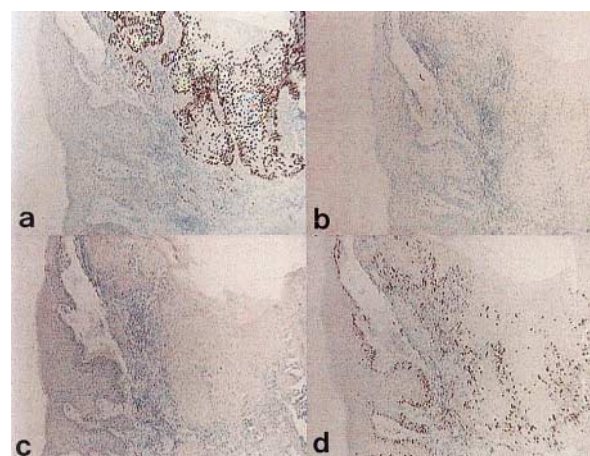


Fig. 2. Biopsy samples taken from keratoacanthoma and stained for (a) p53, (b) p21, (c) PCNA and (d) Ki-67. Intense expression is seen for p53 in the whole epidermis of the lesion. Adjacent epidermis is negative (a). No staining is seen for p21 in KA (b). Only a few scattered cells are positive for PCNA. Ki-67 shows intense expression in basal and suprabasal cells of the lesion. (Original magnifications 40 ×).

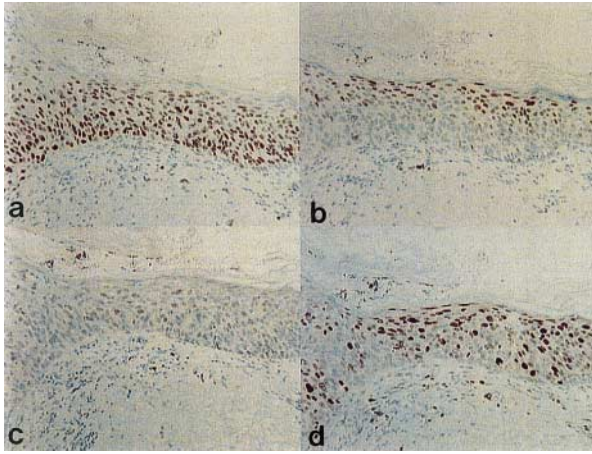


Fig. 3. Actinic keratosis expresses (a) p53 intensely in the whole epidermis, while (b) p21 is detected in the upper part of the epidermis. (c) Immunopositivity for PCNA is much weaker and scattered than for other markers. (d) Ki-67 is intensely expressed in the whole epidermis. (Original magnifications 100×).

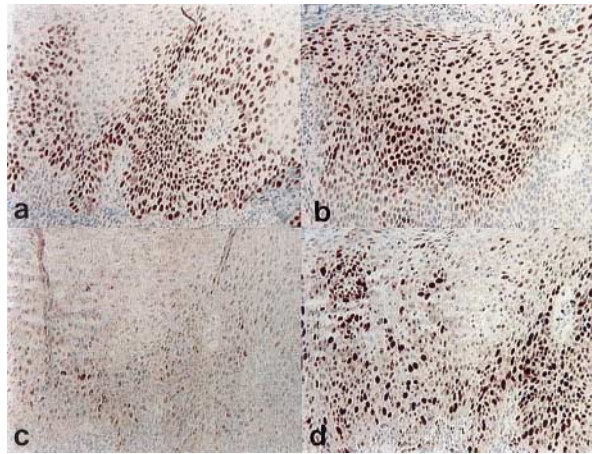


Fig. 4. Extragenital Bowen's disease showing (a) intense immunopositivity for p53. The signals are located in basal and suprabasal cells. (b) p21 expression is strong and located in whole epidermis except the epidermal surface and basal cells. (c) Weak to moderate staining for PCNA is found in most of the epidermal layers, while Ki-67 is intensely expressed in the same region as p53. (d) Ki-67 positive cells are seen even in the upper part of the epidermis. (Original magnification 40×).

found beneath the layers where abundant HPV DNA was present (Figs. 1a, b). The expression of p21 protein colocalized with the HPV DNA signals in the VV specimens, showing intense ISH signals and immunostaining in the upper layers of the epidermis, with no staining in the basal cells (Figs. 1a, c). Ki-67 was expressed intensely in all verrucas analysed (Fig. 1d).

Keratoacanthoma

The staining patterns of cell cycle proteins was totally different in KA and VV, although both are benign lesions. Moderate to intense immunopositivity for p53 was found in 83.3% of KA samples. p53 was detected in the entire

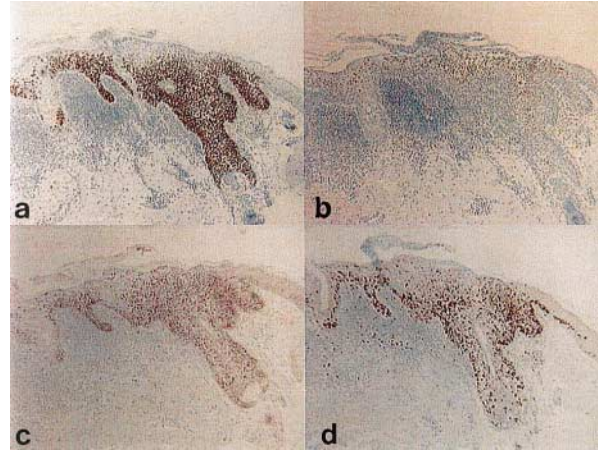


Fig. 5. Squamous cell carcinoma showing intense expression for p53 in the whole epidermis except the surface area. (a) No expression is detected in the normal adjacent epithelium. (b) p21 immunopositivity is detected in the upper third of the epidermis. (c) The staining pattern of PCNA is the same as detected for p53. (d) Ki-67 is expressed lower in the epidermis than PCNA. Adjacent normal epithelium shows intense Ki-67 expression in the basal cells. (Original magnification 40×).

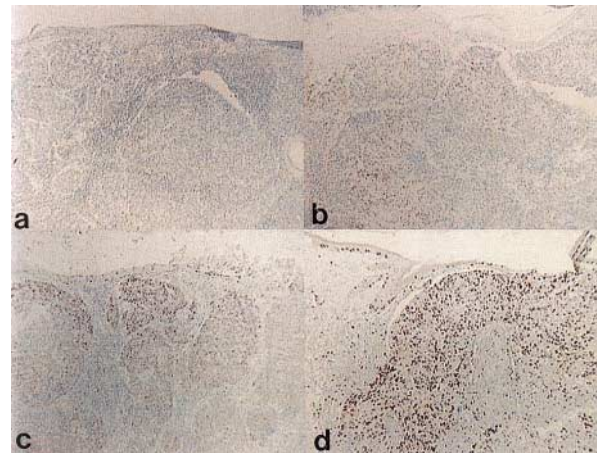


Fig. 6. Another staining pattern of cell-cycle markers found in SCCs. (a) No p53 expression is detected while (b) scattered cells are positive for p21. (c, d) PCNA and Ki-67 both show nuclear staining in cell clusters, focal nuclear staining. (Original magnification 40×).

epidermis except the surface (Fig. 2a). PCNA expression was as low as that of p21 (Figs. 2b, c), while moderate to strong Ki-67 expression was detected in 83.3% of the samples (Fig. 2d).

Actinic keratosis

AKs showed moderate to intense expression for p53 in 79.2%, and the staining was associated with moderate to strong expression of Ki-67 (Figs. 3 a, d). Both of these markers had a similar staining pattern where the signals were detected in the whole thickness of the epidermis. Immunoreactivity for p21 was intense or moderate in only 6 AK samples (25%) (Fig. 3b). The signals were found most frequently in the

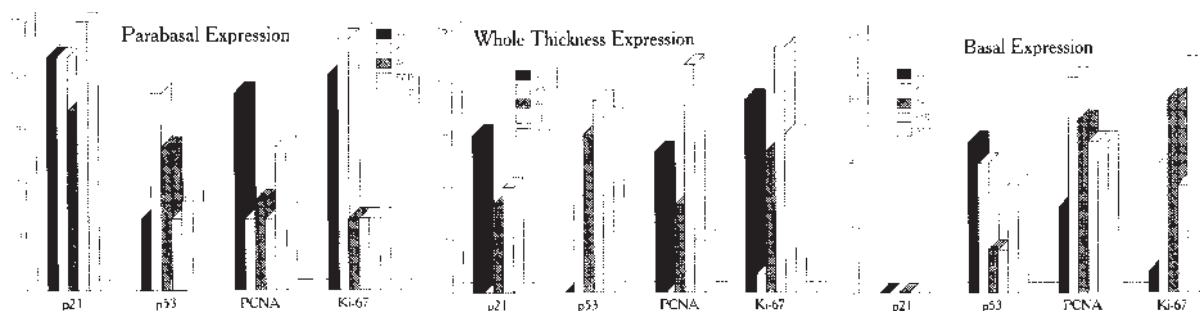


Fig. 7. Expression pattern of p21, p53, PCNA and Ki-67 in different skin lesions (number of patients).

surface of the epidermis. Both weak and intense staining of PCNA was detected (Fig. 3c).

Extragenital Bowen's disease

In EBD lesions, all cell-cycle markers p53, p21, PCNA as well as Ki-67 were overexpressed. The topography of expression resembled that of AKs (Figs. 4a–d). The p21 protein was weakly expressed in the nuclei of suprabasal and lower intermediate layer keratinocytes (Fig. 4b). No p21 expression was found in the basal cells, and no cytoplasmic staining was detected in any of the specimens. Moderate PCNA staining was detected in most of the EBD lesions (Fig. 4c). Intense staining of Ki-67 was demonstrated throughout the whole epithelial thickness (Fig. 4d).

Squamous cell carcinoma

The signal intensity of p53 in SCCs showed a wide variation (Figs. 5a, 6a). Tumour grade was unrelated to p53 staining in the lesions (GI, 60.0%; GII, 83.3%; GIII, 71.4%). The 9 cases of SCC that had strong p53 staining showed a moderate expression of p21 protein only in 3 cases. The expression of p21 protein was similar in both well and poorly differentiated SCCs. No association of p53 expression with cell proliferation activity was found in SCCs, because both p53-positive and p53-negative tumours showed high levels of Ki-67 (Figs. 5d, 6d). Moderate or intense PCNA staining was also found in the nuclei of the vast majority of SCCs, regardless of their differentiation (Figs. 5c, d).

The expression intensity of the cell-cycle proteins in different lesions was separately analysed. p21 protein was moderately or intensely expressed exclusively in VV and EBD lesions, in 16/24 (66.7%) and 18/24 (75%) of cases, respectively. In KA, AK and SCC lesions, the majority showed weak expression only. The expression of p53 was dramatically different from that of p21. Indeed, p53 was moderately or intensely expressed in the vast majority of lesions, except for VV, where only 7/24 (29.2%) of the lesions showed moderate to intense staining.

EBD lesions differ from the others in that almost all, 22/24 (91.7%) lesions express PCNA with moderate or strong intensity. On the other hand, such a strong PCNA staining was rare in KA lesions (6/24, 25%). The lesions did not markedly differ in their Ki-67 expression, while the vast majority of all lesions showed Ki-67 in abundance.

Fig. 7 depicts the different expression patterns (basal, parabasal, full-thickness) of p21, p53, PCNA and Ki-67 in

the lesions studied. It is clearly evident that these different diseases markedly differ in their expression patterns of these cell-cycle proteins. No basal cell expression was established for p21 in any of the lesions. Basal cell expression of p53 was most marked in VVs, and significantly less in AK and EBD lesions. For PCNA, the basal staining was least frequent in VV lesions and more abundant in the others. The difference was even more accentuated for the basal expression of Ki-67. In the parabasal expression, the different lesions were most markedly distinguished by the Ki-67. Ki-67 was abundant in both benign lesions (KA, VV), as contrasted to premalignant (AK, EBD) and malignant proliferations (SCC), where this pattern was rarely encountered.

Concerning the full-thickness expression, some interesting differences were observed. KA seems to differ from the other lesions (including the benign VVs) in its full-thickness expression of p21 (0%), PCNA (0%) and Ki-67 (1/24). On the other hand, no such pattern for p53 was found in VV lesions. EBD seems to peak in the full-thickness expression of both p53, PCNA and Ki-67.

Normal skin

PCNA staining was found only in a few basal cells; Ki-67 protein was detected in the basal layer and in a few parabasal cells; no staining of p53 was found.

DISCUSSION

This study is the first systematic analysis of skin lesions (benign, premalignant and malignant) for the expression of the cell-cycle proteins, p21, p53, PCNA and Ki-67, and at the same time, the first study where the expression of p21 protein has been assessed in any skin lesions. In our series, nuclear p21 labelling was found only in a few basal cells of the normal skin. By contrast, p21 expression (suprabasal or full-thickness) was elevated in almost half of the lesions, including both benign (VV), premalignant lesions (EBD) and skin cancer. Overexpression of p21 protein in these proliferating lesions was unexpected because a major function of p21 is to inhibit the CDK activity (33). Furthermore, p21 protein may also block the function of PCNA and couple the p53 activation to decreased cell proliferation (14, 15). In VV, both p21 expression and HPV DNA signals (by ISH) were found with equal intensity and patterns, characterized by stronger staining in the upper layers of the epidermis than in the deeper levels.

In HPV DNA-positive skin warts, PCNA staining was

extensively present in the epidermis. Similarly, the expression of another proliferation marker, Ki-67, was also found in the VV specimens with a predilection to the suprabasal layers. All VV lesions were positive for HPV 2 DNA, consonant with the highly proliferative character of these lesions. According to the current concepts, the replication of HPV DNA in such lesions is thought to start in the spinous cell layers which have ceased cell cycling (34, 35). Therefore, replication of HPV DNA must rely on the host DNA replication machinery, including the PCNA and Ki-67. Our results are in alignment with this reasoning. The demonstration of abundant HPV DNA signals in the uppermost layers of the epidermis in VV lesions imply that there might be a mechanism by which HPV can reactivate the host genes to support its own DNA replication (36, 37). In such a model, overexpression of p21 in proliferative skin warts might provide a feedback control mechanism used by HPV DNA and other proliferation proteins in an attempt to down-regulate excessive cell proliferation. It was recently demonstrated that the cdk-inhibitory function of p21 is regulated stoichiometrically and p21 inhibits the CDK activities only when it is in molar excess (38, 39).

In the present study, Ki-67 expression was abundant in VV lesions in the suprabasal layers where HPV DNA labelling was not present but PCNA staining was also localized (Fig. 7). This is in accordance with the results reported by Boon et al. (40). PCNA protein is known to be induced during the cell-cycle transition from the G0 to G1 phase and further increased during the S phase (18–20). Ki-67 is present in the G1, S, G2 and M phases of the cell-cycle, but absent in G0 cells (2). In general, Ki-67 immunoreactivity has been shown to bear a close correlation with the PCNA staining (41). Indeed, in VV lesions, the expression pattern of these 2 proteins was quite similar, i.e. suprabasal (Fig. 7).

Half of the biopsies from the SCCs (both well and poorly differentiated tumours) displayed p21 positivity with heterogeneous staining patterns. Such an observation was unexpected because p21 is a known inhibitor of the CDK activity, which in turn stimulates the cell-cycle progression. Jung et al. showed that the level of p21 expression was elevated in 17% of the patients with an acute myelogenous leukaemia compared with the levels in the normal bone marrow (10). They further showed that if the ratio of p21 to cdk is less than one, p21 serves only as an assembling factor for the cdk complex and does not inhibit the CDK activity (38, 39). All these data suggest that the regulation and inhibitory functions of the CDK inhibitor family members (including p21) might vary. In our study, no apparent difference was found in the p21 overexpression between the low-grade and high-grade SCCs. Thus, the p21 protein elevation in SCCs might be an early event in SC carcinogenesis and not directly associated with the progression of malignancy (10). This is further substantiated by our finding of the intense p21 protein staining most frequently (18/24) in (precancer) EBD lesions.

UV radiation, as one of the carcinogenic factors in the skin, is thought to cause cellular abnormality at the DNA level (42). The strong expression of p53 protein in 19/24 AKs which were excised from the sun-exposed skin sites, implies that the defective p53 gene occurred as a consequence of UV. As with the p21 immunoreactivity in SCCs, cancer differentiation was unrelated to the expression of p53 protein in this study. This is in contrast to the findings of Nylander et al. (43), who suggested a correlation between p53 expression and the stage of SCC. These discrepant findings are probably due to the

different anatomic sites of origin of the tumours studied. Tumour site, in this case, may be a more important factor for p53 expression than the clinical tumour stage (43). No association of p53 expression with cell proliferation activity was found in this study because both p53-positive and p53-negative tumours showed high levels of Ki-67. This is consonant with the previous study of Szekeres and Giacomoni (44), but again in contrast to the results by Nylander et al. (43), where localization of p53 and PCNA were similar and p53 levels were increased in areas with proliferative activity.

Skin cancer, as other neoplastic diseases, is a proliferative disorder characterized by uncoordinated cell growth. The p53 protein (wt p53), as an important tumour-suppressor gene in normal cell, effects on moderating cell proliferation. p53 activation leading to decreased cell proliferation can be mediated by WAF-1/Cip1/p21 gene through blocking the initiation of DNA-replication by inhibiting the cyclin-cdk complex. The latter may also block the action of PCNA, a marker of cell proliferation. Immunoreactivity of Ki-67 is also correlated with cell proliferation. HPV has been implicated in the aetiology of cutaneous diseases, including benign, premalignant and malignant. Both UV radiation and HPV infection can induce defects in the p53 gene. The loss of p53 function or mutant forms of p53 are associated with cell proliferation and neoplastic transformation.

The present study showed that the cell-cycle proteins p53, p21 (WAF-1), PCNA and Ki-67 in benign, premalignant and malignant skin lesions have different expression patterns and intensity. Benign (KA and VV) lesions could not be readily distinguished from premalignant (AK, EBD) and malignant lesions on the basis of cell proliferation as measured by Ki-67 staining. Using another proliferation marker (PCNA), however, AK, EBD and SCC did show a more intense staining than KA, but not VV. This indicates that Ki-67 and PCNA measure different aspects of cell proliferation, the latter being a better discriminator of cancer/precancer skin lesions from the benign KA. This might also have some practical implications, e.g. in making the sometimes difficult distinction between KA and SCC. Needless to say, such differential diagnostic difficulties rarely arise between SCC and VV.

Interestingly, the benign KA and VV lesions seem to differ dramatically in their expression pattern and intensity of the cell-cycle proteins p21, p53 and PCNA (but not Ki-67). This implicates that VV but not KA retain the high proliferative activity as measured by PCNA. The most feasible explanation for this difference might be offered by HPV, regularly present in the former, but only exceptionally detected in KAs as yet (28). More studies are needed, however, before final conclusions can be drawn about the cell-cycle proteins in skin lesions with implicated HPV involvement. Of special importance would be prospective cohort (follow-up) studies of the patients with these benign and premalignant skin diseases, to examine any newly appearing lesions as well as the regressing ones for their HPV status and expression of the cell-cycle proteins.

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