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Sun-exposure- and aging-dependent p53 protein accumulation results in growth advantage for tumour cells in carcinogenesis of nonmelanocytic skin cancer

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Abstract Three hundred and sixteen patients with nonmelanocytic skin cancer, including 46 cases of Bowen's disease (BOD), 134 cases of squamous cell carcinoma (SCC), and 136 cases of basal cell carcinoma (BCC), were examined immunohistochemically using monoclonal antibody DO-7 to assess p53 protein accumulation related to sun exposure and ageing, and growth and differentiation of skin cancer and its precursors. The rates of p53 immunostaining of BOD, SCC and BCC were 80.4%, 76.1% and 70.6%, respectively. p53-positive cells were present not only in cancer nests, but also in dysplastic and even morphologically normal epidermis adjoining cancers. Sun exposure was statistically correlated with the p53 immunostaining scores in morphologically normal epidermis of the three skin cancers and in cancer nests of SCC and BCC. The positivity and score of p53 protein often differed significantly among the three types of cancer, especially in regions of dysplasia. Interestingly, differentiation of SCC was correlated with individual p53 scores for dysplasia and cancer nests, especially for dysplasia. BOD, as the precursor of SCC, demonstrated the strongest p53 expression. Furthermore, 12.3% cases with p53 negative cancer nests showed p53-positive reaction in dysplasia and in morphologically normal epidermis. It seems that the accumulation of p53 protein plays a part in precancerous lesions and in the genesis of more highly differentiated types of skin cancer and affects mainly the growth of tumour cells rather than their differentiation. For BCC, however, age was significantly related to p53 expression. Our findings suggest that overexpression of p53 in normal skin and cancer nests of SCC and BCC is significantly related to sun exposure, that the expression of p53 in BCC is an age-dependent process, and that the early accumulation of p53 protein may be a useful predictor for the detection of nonmelanocytic skin cancer.

Key words Skin cancer · p53 · Differentiation · Sun exposure · Ageing

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Introduction

p53 is a tumour suppressor gene and encodes a nuclear phosphoprotein with a short half-life. It has been generally accepted that *p53* is a component in the biochemical pathways central to human carcinogenesis [12], acting as a 'guardian of the genome' by preventing cells bearing damaged DNA from proliferating, either temporarily, by arresting the cell division cycle until damage is repaired, or permanently, by pushing the damaged cell down an irreversible apoptotic pathway of cell suicide [25]. In the absence of functional wild-type *p53*, this function is lost; cells accumulate genetic damage and exhibit marked genetic instability, often to the extent of gross aneuploidy [17]. *p53* gene alterations were found in 40–45% of cases of the ten most frequent cancers in humans [35]. In nonmelanocytic skin cancer, the incidence of which has been increasing for at least two decades, *p53* is the gene most often mutated [22, 31]. About 80% of *p53* mutations are missense mutations, which express aberrant p53 protein with a long half-life, resulting in intracellular overaccumulation of these proteins [16, 19]. A correlation has been demonstrated between the overexpression of p53 protein in tumour cells and the presence of a missense mutation in the *p53* gene [1, 18, 19].

It has been suggested that there is remarkable tissue- and cell-type-specific regulation of the *p53* pathway [15]. In contrast to some tumours, *p53* alteration is an early event in skin cancer [3]. Identification of the early *p53* alteration may greatly facilitate the early diagnosis of skin cancer and provide clues to its genesis. In this study we used a large sample to compare the p53 expression in Bowen's disease (BOD), squamous cell carcinoma (SCC), and basal cell carcinoma (BCC), and also in

morphologically normal epidermis and dysplastic lesions adjoining these cancers, to determine how these expressions are related to clinicopathological factors. Our findings suggest that aberrant p53 expression in the carcinogenesis of different skin cancers has different roles in the causation of tumour cell growth advantage.

Materials and methods

Surgically resected tissues from 316 cases in total were obtained from the Department of Pathology (184 cases) and Clinical Laboratory Medicine of Kochi Medical School (98 cases) and Matsuyama-Shimin Hospital (34 cases) up to 1997, including 46 cases of BOD, 134 of SCC and 136 of BCC.

Specimens were fixed in buffered formalin and embedded in paraffin. Dewaxed paraffin sections were stained with haematoxylin and eosin (H&E). In each case the diagnosis checked by three expert pathologists.

Different sites were graded on a scale of four for exposure to sunlight: 0 (no sun exposure even in swimsuit), 1 (only exposed in swimsuit), 2 (only exposed in a summer suit) and 3 (exposed even in winter suit). Grades 0 and 1 were termed weak sun exposure and grades 2 and 3, strong sun exposure.

From pathological observations, SCC was classified into well, moderately and poorly differentiated subtypes, and BCC into adenoid, solid and other subtypes.

The following variables were recorded, and some mean values were calculated (Table 1): sex, age at the time of diagnosis, degree of sun exposure and pathological diagnosis, including subtypes (not shown).

For immunohistochemical staining, autoclave pretreatment was used for dewaxed paraffin sections prior to incubation with anti-p53 protein monoclonal antibody (DO-7, 1:30 dilution, Dako, Glostrup, Denmark), followed by the application of the labelled streptavidin biotin (LSAB) method (LSAB Kit, Dako). A case with p53 protein staining in gastric cancer was used as a control.

Immunohistochemical staining of p53 was scored by positivity (0: less than 1%, 1: 1–10%, 2: 10–50%, 3: more than 50%) and intensity (0: negative, 1: weak, 2: moderate, 3: strong). The sum score (positivity plus intensity) was then calculated.

The sites observed in each case include cancer nests, and morphologically normal epidermis and dysplastic lesions also if available.

DNA was extracted from microdissected dewaxed paraffin sections for each of 10 positive or 10 negative cases for p53 protein accumulation. After PCR amplification of *p53* exons 5–8, SSCP analysis was employed to screen for *p53* mutation. Direct sequencing was then performed for mutation point analysis of the DNA samples that exhibited altered mobility on SSCP analysis.

Table 1 p53 Expression in 316 cases of nonmelanocytic skin cancer and relevant statistical analysis (*BCC* basal cell carcinoma, *BOD* Bowen's disease, *SCC* squamous cell carcinoma, *NS* no significance, i.e. R<0.20 or *P*>0.05)

	Item	BOD	SCC	BCC	Total
Clinical features	No. of cases	46	134	136	316
	Sex (male/female)	22/24	72/62	84/52	178/138
	Age				
	Average	71.1	79.0	71.6	74.7
	Range	41–88	24–105	34–95	24–105
	Sun exposure				
	Average sun exposure grade	1.500	2.567	2.529	2.396
Sum score of p53 expression	Morphologically normal skin				
	Positive rate (%)	4/36 (11.1)	28/75 (37.3)	31/102 (30.4)	63/213 (29.6)
	Average sum score	0.361	1.293	0.931	0.962
	Dysplasia				
	Positive rate (%)	6/19 (31.6)	47/61 (77.1)	34/97 (35.1)	87/177 (49.2)
	Average sum score	1.316	3.180	1.247	1.921
	Cancer nest				
	Positive rate (%)	37/46 (80.4)	102/134 (76.1)	96/136 (70.6)	235/316 (74.4)
	Average sum score	3.739	3.209	2.346	2.915
Statistical analysis	p53 expression				
	Normal skin and dysplasia				
	Difference in % (P[a])	NS	<0.01	NS	<0.01
	Difference in sum score (P[b])	<0.05	<0.01	NS	<0.01
	Correlation (R/P[c])	0.70/<0.01	0.54/<0.01	0.24/<0.05	0.41/<0.01
	Dysplasia and cancer nest				
	Difference in % (P[a])	<0.01	NS	<0.01	<0.01
	Difference in sum score (P[b])	<0.05	NS	<0.01	<0.01
	Correlation (R/P[c])	NS	NS	0.29/<0.01	0.35/<0.01
	p53 expression and sun exposure				
	Morphologically normal skin	NS	0.21/<0.05	0.31/<0.01	0.31/<0.01
	Dysplasia	NS	NS	NS	NS/<0.05
	Cancer nest	NS	0.22/<0.05	0.23/<0.01	NS
	p53 expression and age				
	Morphologically normal skin	NS	NS	0.23/<0.01	0.20/<0.01
	Dysplasia	NS	NS	0.20/NS	0.28/<0.05
	Cancer nest	NS	NS	0.26/<0.01	NS/<0.05

[a] *P* stands for *P* value from chi-square test for positive rate (%)
[b] *P* stands for *P* value from paired *t*-test for score
[c] In R/P, R means the correlation value by Pearson's correlation coefficient; *P* means the correlation value from Spearman's correlation coefficient by rank

Methods used for statistical analysis included: determination of Pearson's correlation coefficient and Spearman's rank-correlation coefficient for correlation analysis, chi-square test for analysis of difference between rates of positivity, paired *t*-test for analysis of differences between paired groups, and Mann-Whitney's U test for analysis of difference between unpaired groups.

Results

More than two-thirds of the cases examined were positive for p53 in cancer nests (Table 1). In p53-positive SCC cases, especially those that were well-differentiated, brown-stained nuclei were present in the periphery of cancer nests (Fig. 1A). In BOD, the positive-stained nuclei were commonly dark brown, diffusely present through the entire thickness of epithelium replaced by neoplastic cells (Fig. 1B). In BCC, the distribution of positive cells was also diffuse, but often predominated in the periphery of cancer nests with palisade-arranged cells (Fig. 1C). Positive cells were found not only in cancer nests (Fig. 1A–C) but also in dysplastic and even morphologically normal epidermis adjoining BOD, SCC and BCC lesions (Fig. 1D, Table 1).

Of 20 DNA samples from dewaxed tissues, no mutation was found in any of the 10 cases negative for p53 protein immunohistochemical staining. Mutations were found in 2 of the 10 cases positive for p53 protein staining. In 1 of these, a case of BOD, a C→T transition (Pro→Leu) occurred in codon 278 of exon 8. In the other, a case of SCC, a G→A transition (Arg→Gln) was found in codon 248 of exon 7.

Rates of positivity and average sum scores for p53 expression in nonmelanocytic skin cancer are shown in Table 1. For the group of all 316 cases, the rate of positivity and average sum score tended to increase from morphologically normal epidermis, through dysplasia, to cancer nests. Statistical analysis also demonstrated differences and correlations in p53 expression both between morphologically normal epidermis and dysplasia, and between dysplasia and cancer nests. Among the three kinds of skin cancer, rates of positivity and average sum scores for p53 expression in cancer nests were highest for BOD (Fig. 1A–C, Table 1). In dysplastic skin, positivity and sum score were significantly higher for SCC than for BCC or BOD.

Fig. 1 **A–D** Immunohistochemical staining of p53 in Bowen's disease (BOD), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). **A** Strong p53 positivity in the periphery of cancer cell nests of a well-differentiated SCC on the face of an 80-year-old woman. ×200. **B** Diffuse p53-positive staining in BOD in the lower abdominal wall of a 73-year-old man. ×200. **C** Predominant p53 positivity of peripheral palisade cells of BCC, solid type, on the nose of a 78-year-old woman. ×125. **D** Strong p53 positivity in the cancer cell nests (*upper left*) and moderate positivity in the adjoining dysplastic regions (*down and to right*) in well-differentiated SSC on the neck of an 82-year-old man. ×100

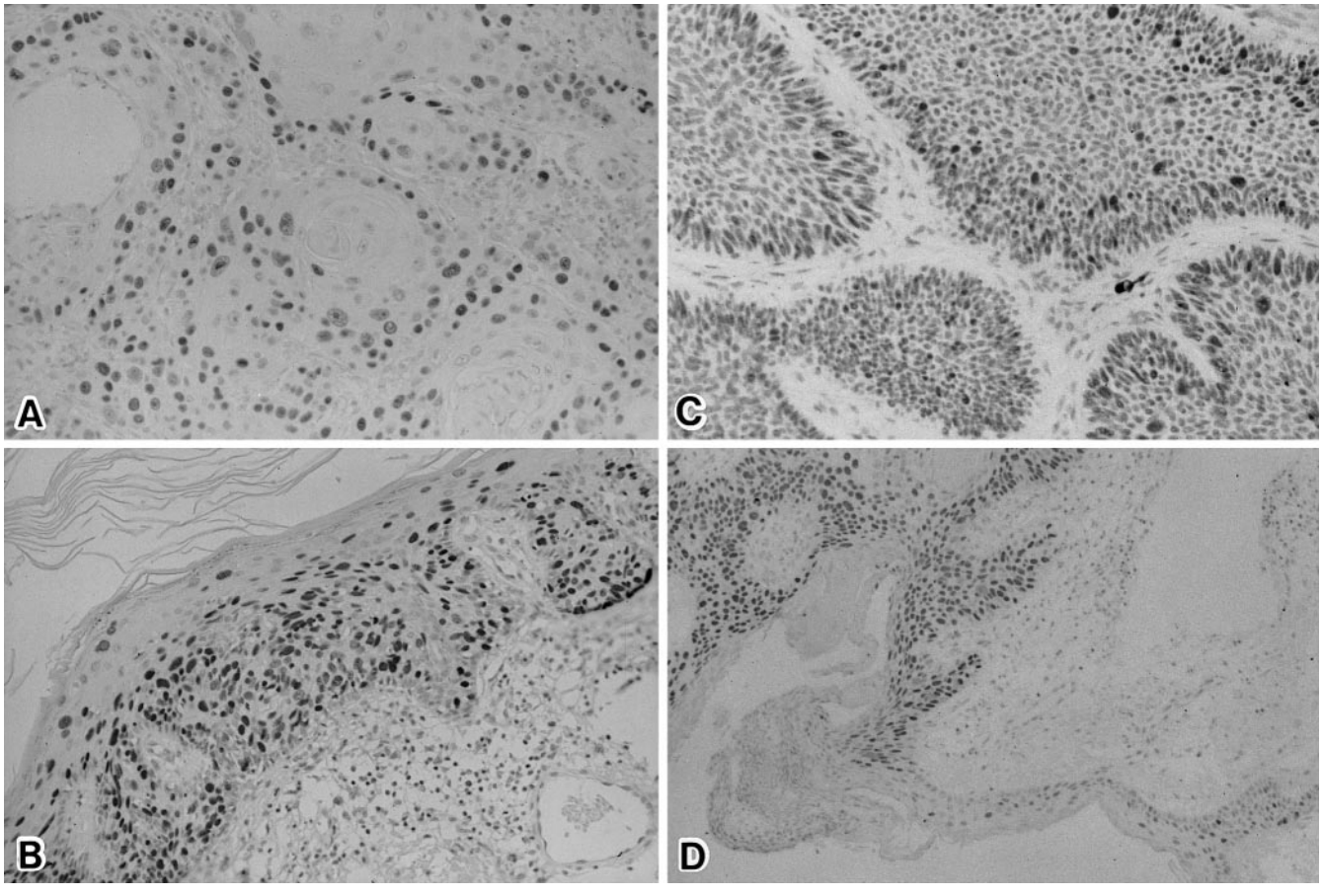

Table 2 Comparison and statistical analysis between different types of skin cancer for age, sun exposure and p53 expression

Tumours	Comparison and statistical analysis (P[a])					
	Age	Score for sun exposure	Positive rate and score for p53 expression			
			Item	Normal skin	Dysplasia	Cancer
BOD vs SCC	<0.01	<0.01	%	<0.01	<0.01	NS
			sum score	<0.01	<0.01	NS
SCC vs BCC	<0. 01	NS	%	NS	<0.01	NS
			sum score	NS	<0.01	<0.01
BCC vs BOD	NS	<0. 01	%	<0.05	NS	NS
			sum score	<0.05	NS	<0.01

[a] *P*-values is calculated by the Chi-square test for positive rate (%), and by Mann-Whitney's U test for score (*NS* no significance, i.e. $P>0.05$)

Among 81 cases with p53-negative cancer, 10 cases (12.3%), 1 case of BOD, 4 cases of SCC and 5 cases of BCC, exhibited p53 positivity in dysplasia and even in morphologically normal epidermis.

A total of 76.6% (242/316) of skin cancers were found in strongly sun-exposed areas. On the whole, sun exposure was related to p53 expression in noncancerous skin adjoining cancer nests, especially in morphologically normal epidermis (Table 1). In SCC and BCC, p53 immunostaining scores for morphologically normal epidermis were correlated with sun exposure. Furthermore, the grades for sun exposure in morphologically normal epidermis of SCC and BCC were significantly higher than that for BOD, and so were the scores for p53 expression (Tables 1, 2). In BOD, the rate of positivity and sum score for morphologically normal epidermis were also much higher in the strong sun exposure group (38.5%, 0.692) than in the weak sun exposure group (13.0%, 0.174), although the number of cases was too small to demonstrate a significant correlation. For cancer nests in the group of all cases, although no significant correlation was found between grade of sun exposure and score for p53 expression, the rate of positivity and sum score were clearly higher in the strong sun exposure group than in the weak sun exposure group ($P<0.05$ and $P=0.05$). In addition, a correlation of sun exposure with p53 expression was found for the cancer nests in the cases of SCC and BCC, especially the well-differentiated subtype of SCC (R/P=0.34/0.02). For BOD, however, the sum score of p53 expression of cancer nests (3.964) in the weak sun exposure group (sun exposure grade was 0.857) was highest among the various subtypes of skin cancers, including the strong sun exposure group of BOD (sum score of p53 expression was 3.389, sun exposure grade was 2.500), although a statistical comparison between the two groups was not possible owing to the small number of cases.

Overall, age was related to score for p53 expression, especially for dysplasia (Table 1). Among individual diseases, however, this relationship was only significant for BCC.

For SCC, a correlation between p53 score and differentiation was present in dysplasia (R/P=0.51/<0.01), and a less evident correlation in cancer nests (no/<0.05). Thus, the more differentiated the tumour, the higher the p53 score. The sum score for cancer nests was significantly higher in the well-differentiated SCC group than in the moderately and poorly differentiated groups ($P<0.05$). For BCC, the sum score for p53 expression in dysplasia and cancer nests was predominant in the adenoid type as opposed to the solid type (Fig. 2), although not to a significant extent.

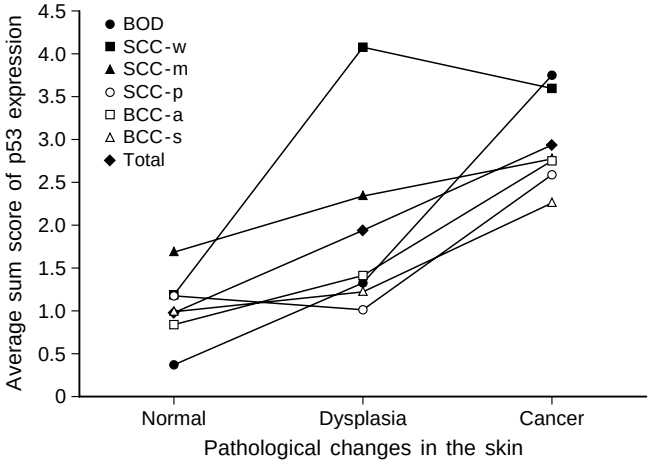

Fig. 2 Comparison of p53 expressions among various subtypes in 307 cases of skin cancer (*SCC-w*, well-differentiated SCC, *SCC-m* moderately differentiated SCC, *SCC-p*, poorly differentiated SCC, *BCC-a*, adenoid type of BCC, *BCC-s*, solid type of BCC)

Discussion

Mutation of the *p53* gene has been found to play important parts in various types of malignancies including skin cancer [12]; *p53* gene point mutations lead to the production of proteins that are more stable than their wild-type counterparts, resulting in intracellular overaccumulation of such proteins [19]. Alterations in the *p53* gene and/or its protein product frequently occur as late events in multistep carcinogenesis. For example, p53 may play a functional role in development of the metastatic phenotype of malignant melanomas [36]. However, recent studies have indicated that *p53* alteration may occur as an early event in SCC of the upper aerodigestive tract [7], head and neck [33], larynx [10], bronchus [2] and

oesophagus [13], as well as in adenocarcinoma of the lung [26] and oesophagus [37]. In skin cancer, *p53* mutation is a late event in chemically induced cases [6, 32], whereas it is an early event in cases following chronic UV exposure [3]. We compared p53 expression in tumour nests and noncancerous epidermis adjoining them for nonmelanocytic skin cancers, including SCC, BCC and BOD. For the entire group of 316 cases, positive cells were found in 74.4% (235/316) of tumour nests, in 29.6% (63/213) of morphologically normal epidermis samples and in 49.2% (87/177) regions of dysplasia adjoining skin cancers. Histopathologically, cells positive for p53 protein are usually present in the periphery of cancer nests of SCC and BCC. This supports the suggestion that overexpression of p53 begins in the early stage of carcinogenesis before the appearance of malignancies in skin, and may be a predictor of skin cancer. Coupled with knowledge of the significant differences in age and sun exposure in the cases with different kinds of skin cancer (see Table 2), the early expression of p53 in noncancerous skin may help us to predict progression through dysplasia to carcinoma and the possible histopathological pattern of skin cancer (Fig. 2).

In morphologically normal epidermis, the score for p53 expression was significantly higher in SCC and BCC than in BOD cases. This seems reasonable, given the stronger sun exposure of skin with SCC and BCC than that with BOD (Table 2) and the correlation between p53 score and sun exposure grades in morphologically normal epidermis for all three kinds of skin cancer (Table 1). CC to TT mutations in the *p53* gene (specifically induced by UV radiation) were detected in cultured human skin cells only after UV irradiation, and mutation frequency increased in a UV dose-dependent manner [28]. Other studies have shown that *p53* mutations in normal skin accumulate as a result of chronic sun exposure during daily life [3, 21, 30, 40]. Our study provides further immunohistochemical evidence for this relationship between p53 expression in morphologically normal epidermis and sun exposure. For BOD, the rate of positivity and the sum score of p53 expression in morphologically normal epidermis were also much higher in the strong sun exposure group. In addition, the correlation between age and p53 scores for morphologically normal epidermis of BCC may be due in part to higher cumulative sun exposure in older individuals. Thus, the accumulation of p53 protein in normal skin may be closely related to sun exposure.

A total of 76.6% (242/316) of skin cancers were found in strongly sun-exposed areas. Although a significant correlation of sun exposure grade with scores for p53 expression in cancer nests was not found for the group of all 316 cases, the rate of positivity and the sum score were clearly higher in the strong sun exposure group than in the weak one. In addition, a correlation of sun exposure with p53 expression was found for cancer nests of SCC and BCC, especially the well-differentiated type of SCC. These findings indicate that in nests of these skin cancers, sun exposure still affects p53 expression dramatically and also supports the hypothesis that sunlight acts as a tumour initiator and promoter [4, 5, 42]. The biological effects of p53 mutations on keratinocytes are quantitative, rather than all-or-none [42]. Sunlight acts as a tumour promoter by favouring the clonal expansion of *p53*-mutated cells [21], but our findings cannot explain why p53 expression was stronger in the weak sun exposure group of BOD than in any of the various other subtypes of skin cancers, including the strong sun exposure group of BOD. This suggests that sun exposure may not be the only cause of p53 protein accumulation in skin, and that strongly and weakly sun-exposed BOD have different pathogeneses. A recent study proposed that the frequent frameshift mutations observed in Japanese BOD may be caused by exposure to unknown environmental chemical carcinogens other than to ultraviolet light [38]. Arsenic might be one such chemical carcinogen [24].

A significant trend toward increasing *p53* mutation frequency with advancing age was found for the 40 cases of normal skin [30] and the 45 cases of BCC [8]. In our study, only BCC exhibited a marked correlation between p53 expression and patient age. According to the somatic mutation theory, ageing phenotypes are the result of an accumulation of somatic mutations in the body [27]. The age-dependent p53 expression of BCC underlines the role of the ageing process in p53 overexpression. In addition, this may be related to the higher cumulative sun exposure in older individuals [3, 21]. Furthermore, some researchers believe that photodamage may be more slowly repaired in aged skin, leading to faster accumulation of lesions and thus to mutations [39–41]. Wild-type p53 might also be induced in response to the increased DNA damage associated with ageing [11, 23].

It has been reported that clonal expansion of *p53*-mutant cells is associated with the histological progression of brain tumours [34]. In renal cell carcinoma, including both carcinomatous and sarcomatous lesions, *p53* mutations are closely associated with sarcomatoid components [29]. Among thyroid carcinomas, *p53* mutations occur almost exclusively in poorly differentiated tumours [20]. These findings suggest a critical role for *p53* alteration in malignant differentiation of carcinomas, but in contrast, we found that p53 expression in dysplasia and cancer nests of skin SCC exhibited a significant positive correlation with differentiation (Fig. 2), especially for dysplasia; the higher the degree of differentiation, the stronger the p53 expression. As in SCC, the p53 score in dysplasia and cancer nests of BCC also tended to be higher in the more highly differentiated adenoid type than in the less well-differentiated solid type. p53 expression appears to be more important in carcinogenesis in the well-differentiated type of skin cancer. The significant difference between morphologically normal epidermis and dysplasia in rate of positivity and score for p53 expression in both SCC and BOD demonstrates a progressive increase in p53 expression from normal skin to dysplasia (Table 1). This increase may be due to the growth advantage obtained by “gain of function” of cells

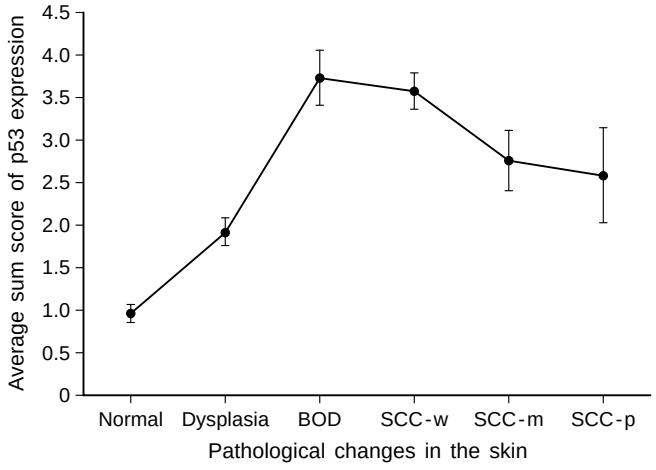

Fig. 3 p53 expression in SCC and its precursor

carrying certain mutant *p53* genes [9]. However, no difference or correlation with p53 expression was found between dysplasia and cancer nests within each type of skin cancer, except for BCC. There were 81 cases with a negative reaction for p53 in cancer nests, and 10 of these showed p53 positivity in dysplasia and in morphologically normal epidermis. This suggests that p53 expression is of less significance during the stage of progression from dysplasia to malignancy. One or more other genes might be important at this stage.

The cancer nests of BOD, the precursor of SCC, displayed the strongest p53 expression of the three types of skin cancer examined. For well-differentiated SCC, the strongest p53 expression was not in cancer nests (sum score was 3.579), but in dysplasia (4.059), where it was even higher than in cancer nests of BOD (Table 1). For the sum score of p53 expression as a measure of the commonly accepted evolution of invasive squamous cell carcinoma from precursor lesions of dysplasia and carcinoma in situ (BOD; Fig. 3), we found that after the peak value in BOD, the sharply ascending curve begins to descend suddenly with more malignant differentiation of SCC, suggesting that in squamous cell malignancies (include BOD and SCC) accumulation of p53 has its main role in progression from normal skin through dysplasia to BOD, and appears mainly to affect the growth of cells but not their differentiation. As shown in Tables 1 and 2 and in Fig. 2, the level of p53 protein accumulation is different in different types of skin cancer and in different stage of oncogenesis. This difference in p53 expression is evident before the establishment of malignancy and is seen mainly in dysplasia. It is thus clear that p53 expression has different roles in the development of BOD, SCC and BCC.

Many researchers have demonstrated a good correlation of *p53* gene mutation detected directly by DNA sequencing and SSCP, with protein overexpression demonstrated by immunohistochemical analysis [1, 18, 19]. In this study, even though *p53* mutation was found in only 2 of the 10 p53-positive cases, no mutation was detected in any of the 10 p53-negative cases. This supports the hypothesis that the accumulation of p53 protein is mainly the result of *p53* gene mutation. The loss of *p53* gene mutation in the other 8 p53-positive cases may have occurred because the samples used for mutation analysis were taken from paraffin-embedded sections, in which DNA might have been damaged or fragmented during preparation of paraffin blocks. It is also possible that the *p53* mutation might lie outside of exons 5 through 8 in rare cases or that SSCP is not sufficiently sensitive to detect all *p53* mutations. Finally, genotoxic insults such as radiation may produce an immunohistochemically detectable wild-type p53 product in normal cells [14, 25] and perhaps also in tumour cells [12]. Further studies are needed to determine the direct relationship of *p53* mutation to carcinogenesis in morphologically normal epidermis and dysplastic or cancerous lesions in skin; these should be performed on fresh tissues, which were not available in the present study.

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