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Overexpression of cyclin D1 in nonmelanocytic skin cancer

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Abstract Although the overexpression of cyclin D1 has been believed to play important roles in neoplastic transformation of some tumors, little is known about the function of cyclin D1 protein in carcinogenesis in human skin. A total of 307 patients with nonmelanocytic skin cancer, being 46 with Bowen's disease (BOD), 134 with squamous cell carcinoma (SCC) and 127 with basal cell carcinoma (BCC), were investigated immunohistochemically using monoclonal antibody to cyclin D1 by the LSAB method, to assess the expression of cyclin D1 in skin cancer including its precursors. The positive rates of cyclin D1 immunostaining in BOD, SCC and BCC were 63.0%, 69.4% and 54.3%, respectively. The positive rates in dysplasia adjoining BOD, SCC and BCC were 43.6%, 67.9% and 59.8%, respectively. In morphologically normal skin, however, only 2 cases, 1 of SCC and 1 of BCC, exhibited positive staining. These findings suggested that overexpression of cyclin D1 is an early event in dysplastic lesions of skin. Overexpression of cyclin D1 was related to sun exposure, especially in dysplasia of SCC. The score for cyclin D1 expression in dysplasia of BCC was correlated with age. Expression of cyclin D1 markedly increased from normal skin through dysplasia to BOD, but was not significantly related to the degree of SCC differentiation. These findings demonstrate that the effect of cyclin D1 overexpression is restricted to proliferation of cells, so that they gain a growth advantage, but their differentiation is not increased. Comparison with the results for p53 protein expression in these tumors, a significant correlation with cyclin D1 expression was found in dysplasia in BOD and SCC, and in patients with BCC who were less than 74 years old. These findings suggested the hypothesis that prior aberrant p53 expression may affect or regulate the overexpression of cyclin D1.

Key words Cyclin D1 · Skin cancer · Differentiation · Sun exposure · Aging

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Introduction

In the cell cycle clock, the transition from G_1 phase to S phase is believed to be the most important checkpoint [11]. As a cell cycle regulator, cyclin D1 forms a complex with CDK4, a cyclin-dependent kinase, to phosphorylate Rb protein, a tumor suppressor gene product, to govern the G_1-to-S transition. Cell cycle regulation is a complex process. Wild-type p53 is thought to be activated when DNA is damaged in the G_1 phase [18]; this increases the amount of p21 and thereby inhibits the activity of cyclin D-CDK4 and cyclin E-CDK2 complexes, thus preventing cells from entering S phase. Overexpression of cyclin D1, which is due to gene amplification, has been found in different types of human malignant tumors, and in breast [10, 23], esophagus [5, 14], liver [34], head and neck [20], lung [28], larynx [13], bladder [16], uterus [22], colorectum [1], and lymphoid tissues [30]. Cyclin D1 has been considered to be an oncogene, and it is believed that it has an important role in the neoplastic transformation of some tumors [11]. Skin cancer has recently become one of the commonest human malignancies. To our knowledge, however, reports on the role of cyclin D1 in skin cancer, and especially on carcinogenesis in human skin are very few. In the present study, we used a large sample to compare the cyclin D1 expression in Bowen's disease (BOD), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) of skin and also in morphologically normal skin and dysplastic lesions adjoining these cancers, and examined relationship of its expression with clinicopathological factors. In addition, we statistically investigated the relationship between cyclin D1 expression and p53 overex-

pression, which has been obtained and reported previously [17]. Our findings suggest that overexpression of cyclin D1 in carcinogenesis of different skin cancers, as a sun exposure- and aging-related factor, plays different parts in order to obtain growth advantage, and that p53 protein may have a key role in the regulation of cyclin D1 expression in skin as an important control factor.

Materials and methods

Patients

Surgically resected tissues from a total of 307 cases were obtained from the Departments of Pathology (175 cases) and Clinical Laboratory Medicine (98 cases) of Kochi Medical School and Matsuyama-Shimin Hospital (34 cases) throughout 1997. These were 46 cases of BOD, 134 of SCC, and 127 of BCC.

Clinicopathologic variables

Specimens were fixed in buffered formalin and embedded in paraffin. Dewaxed paraffin sections were stained with hematoxylin and eosin (HE). The diagnosis was subjected to triple checking by expert pathologists in all cases.

Based on the results of pathologic observation, SCC was classified into well-, moderately and poorly differentiated subtypes, and BCC into adenoid, solid and other subtypes.

By site, degree of sun exposure was divided into four grades: 0 (no sun exposure), 1 (exposed only in swimsuit), 2 (exposed only in summer clothes) and 3 (not covered even in winter clothes). Grades 0 and 1 were also termed weak sun exposure, while grades 2 and 3 were termed strong sun exposure.

The following variables were recorded and mean values were calculated: sex, age at the time of diagnosis, degree of sun exposure (Table 1) and pathological diagnosis, including subtypes (Fig. 4).

Immunohistochemistry

For immunohistochemical staining, pretreatment with autoclaving was used to strengthen the immune reaction before incubation with anti-cyclin D1 mouse monoclonal antibody (NCL-CYCLIN D1-GM, 1:20 dilution, Novocastra, England), followed by application of the labeled streptavidin–biotin (LSAB) method (LSAB kit, Dako, Glostrup, Denmark). A case of cyclin D1-positive bladder cancer was used as a control.

Immunohistochemical staining of cyclin D1 was scored according to the percentage of all tumor cells positive for staining (0 less than 1%, 1 1–10%, 2 11–50%, 3 more than 50%) and intensity of immunostaining (0 negative, 1 weak, 2 moderate, 3 strong). A sum score (positivity plus intensity) was then calculated.

Table 1 Cyclin D1 expression in 307 cases of nonmelanocytic skin cancer and relevant statistical analysis (*BCC* basal cell carcinoma, *BOD* Bowen's disease, *SCC* squamous cell carcinoma)

	BOD	SCC	BCC	Total
Clinical features				
No. of cases	46	134	127	307
Sex (male/female)	22/24	72/62	77/50	171/136
Age				
Average	71.1	79.0	71.6	74.7
Range	41–88	24–105	34–95	24–105
Sun exposure (average grade)	1.500	2.567	2.529	2.396
Sum score of cyclin D1 expression				
Morphologically normal skin				
Positive rate	1/37 (2.7)	1/76 (1.3)	0/91 (0.0)	2/204 (1.0)
Average sum score	0.108	0.066	0.000	0.044
Dysplasia				
Positive rate	17/39 (43.6)	55/81 (67.9)	61/102 (59.8)	133/222 (59.9)
Average sum score	1.692	2.556	2.225	2.252
Cancer nest				
Positive rate (%)	29/46 (63.0)	93/134 (69.4)	69/127 (54.3)	191/307 (62.2)
Average sum score	2.761	3.067	2.220	2.671
Statistical analysis				
Cyclin D1 expression				
Normal skin and dysplasia				
Difference in % (*P*)[a]	<0.01	<0.01	<0.01	<0.01
Difference in sum score(*P*)[b]	<0.01	<0.01	<0.01	<0.01
Correlation (*R*/*P*)[c]	NS[d]	0.28/NS	NA[e]	NS/0.03
Dysplasia and cancer nest				
Difference in %(*P*)[a]	NS	NS	NS	NS
Difference in sum score(*P*)[b]	<0.01	<0.01	NS	<0.01
Correlation (*R*/*P*)[c]	0.53/<0.01	0.37/<0.01	0.38/<0.01	0.40/<0.01
Cyclin D1 expression and sun exposure				
Morphologically normal skin	NS	NS	NA	NS
Dysplasia	NS	0.31/<0.05	NS	NS/<0.01
Cancer nest	NS	NS	NS	NS
Cyclin D1 expression and age				
Morphologically normal skin	NS	NS	NA	NS
Dysplasia	NS	NS	0.35/<0.01	0.22/<0.01
Cancer nest	NS	NS	NS	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
[d] NS means no significance, that is $R<0.20$ or $P>0.05$
[e] Cannot be calculated because not applicable

The sites observed in each case included cancer nests and morphologically normal skin and dysplasia if available.

Statistics

Two calculation methods were used for the analysis of correlation: one was Pearson's correlation coefficient, yielding a correlation value represented by "*R*", and the another one was Spearman's correlation coefficient by rank, yielding a correlation value represented by "*P*". Other statistical analyses included: the Chi-square test for analysis of differences in rates of positivity, the paired *t*-test for analysis of differences between paired groups, and Mann-Whitney's U test or Student's *t*-test for analysis of differences between unpaired groups.

Results

Immunohistochemical staining

The cyclin D1-positive cells show brown-stained nuclei. In BOD (Fig. 1), positive cells were distributed through the entire thickness of the atypical dysplastic epithelium. In SCC, a positive reaction to cyclin D1 antibody was observed in tumor cells (Fig. 2a). In BCC, positive cells were commonly present diffusely in cancer nests (Fig. 3). Morphologically normal skin was usually negative for cyclin D1 immunostaining (Figs. 2c, 3). In dysplasia adjacent to the cancer nests in SCC, many basal and parabasal nuclei exhibited positive immunostaining (Fig. 2b).

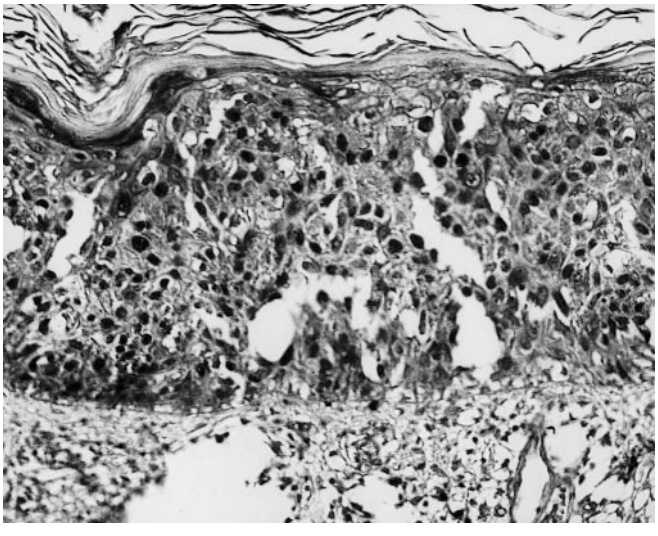
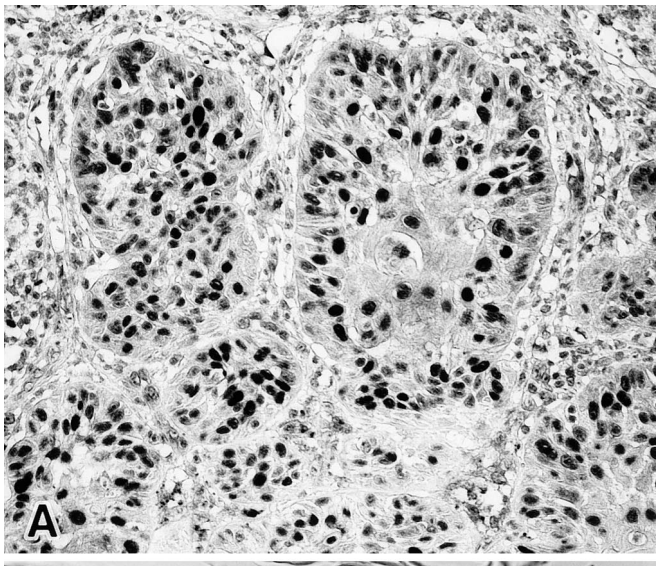
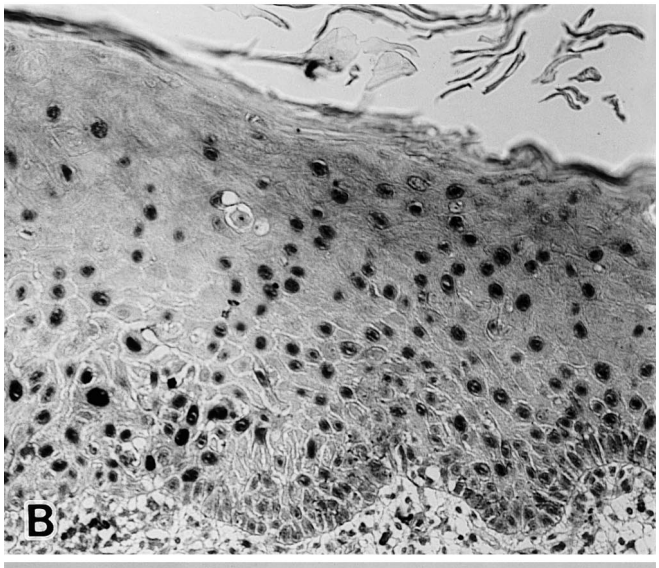


Fig. 1 Bowen's disease in the lower abdominal wall of a 73-year-old man. Cyclin D1-positive staining is present in the entire thickness of the atypical and dysplastic epithelium. Immunohistochemical staining of cyclin D1. ×200





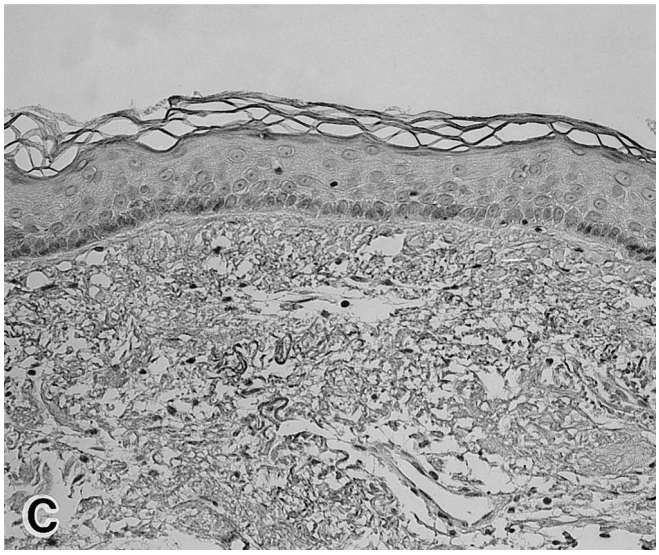


Fig. 2a–c Squamous cell carcinoma in the ear of a 73-year-old man. **a** The nuclei of neoplastic cells show intense reaction to cyclin D1 antibody. ×200 **b** Moderate to strong cyclin D1-positive staining is also observed in many basal and parabasal nuclei of the dysplastic epidermis adjacent to a cancer nest. ×200 **c** Morphologically normal skin shows negative reaction to cyclin D1 antibody. Immunohistochemical staining of cyclin D1. ×160

Table 2 Comparison and statistical analysis between different skin cancer for age, sun exposure and cyclin D1 expression (*P*-values are calculated by Chi-square test for positive rate (%), and by Mann-Whitney's U test for score)

[a] NS (no significance) means $P>0.05$

Tumor	Age	Score of sun exposure	Positive rate and score of cyclin D1 expression			
			Item	Normal skin	Dysplasia	Cancer
BOD and SCC	$P<0.01$	$P<0.01$	%	NS[a]	$P<0.01$	NS[a]
			Sum score	NS	$P<0.05$	<0.05
SCC and BCC	$P<0.01$	NS	%	NS	NS	NS
			Sum score	NS	NS	$P<0.01$
BCC and BOD	NS	$P<0.01$	%	NS	NS	NS
			Sum score	NS	NS	$P<0.05$

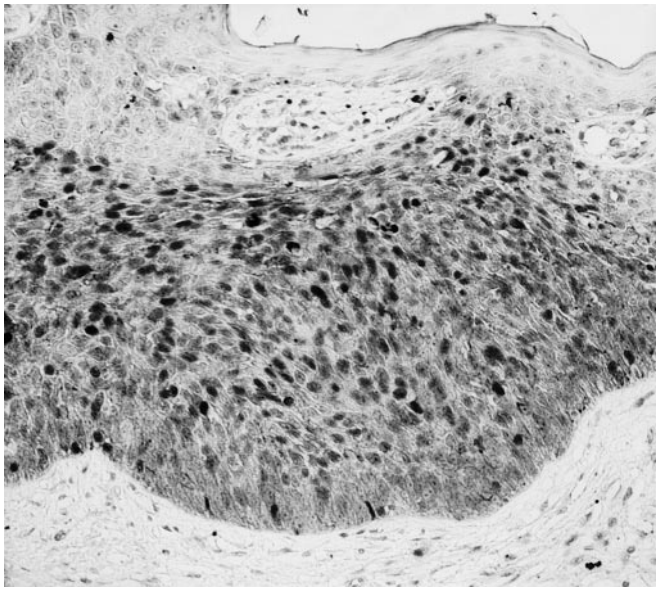


Fig. 3 Basal cell carcinoma in axillary fossa of an 87-year-old woman. The cancer nest shows a strong positive reaction to cyclin D1 antibody, while the morphologically normal epidermis adjacent to cancer nest shows negative reaction. Immunohistochemical staining of cyclin D1. ×200

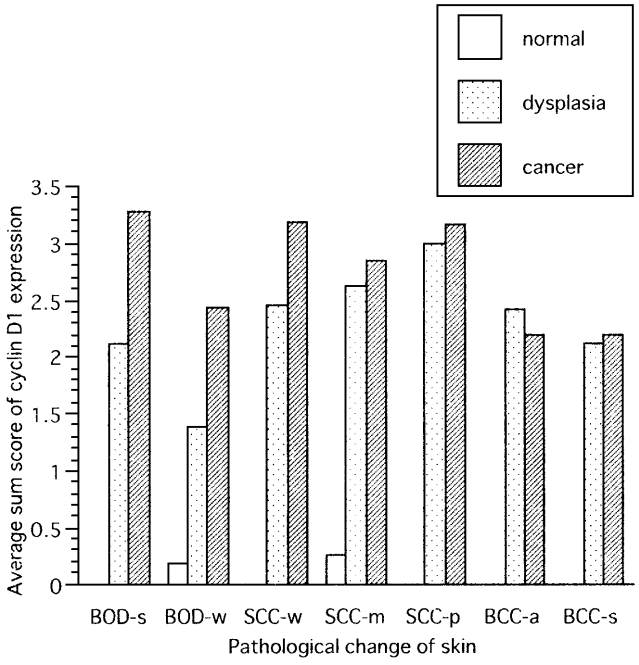


Fig. 4 Comparison of cyclin D1 expression among various subtypes of skin cancer (*BOD-s* strongly sun-exposed group BOD, *BOD-w* weakly sun-exposed BOD group, *SCC-w* well-differentiated SCC, *SCC-m* moderately differentiated SCC, *SCC-p* poorly differentiated SCC, *BCC-a* adenoid type of BCC, *BCC-s* solid type of BCC)

Scores for cyclin D1 expression in morphologically normal skin, dysplasia and cancer nests

The rates of positivity and average sum score of cyclin D1 expression in BOD, SCC and BCC are summarized in Table 1. For the total sample of 307 cases, the rate of positivity and average sum score tended to increase from morphologically normal skin through dysplasia to cancer nests. Only 2 cases of morphologically normal skin, 1 each affected by BOD and SCC (Table 1, Fig. 4), exhibited a positive immunoreaction, mainly in the basal layer. Dysplasia, which is characterized by increased cell growth, presence of atypical morphology and altered differentiation, was sometimes observed adjacent to cancer foci (Fig. 2b). Compared with morphologically normal skin, the number of positive cases increased rapidly in the specimens of dysplasia, up to more than 40%, while the rate of positivity of cyclin D1 expression in cancer nests did not differ significantly from that in dysplasia (Table 1). In BOD, the score for cyclin D1 expression showed a significant increase from dysplasia to cancer nests, and a significant correlation between dysplasia and cancer nests. Although the same significant increase and correlation were also present in SCC, by subtype, these findings were significant only for well-differentiated SCC ($P<0.0001$ and $R=0.391$).

Sun exposure and cyclin D1 expression

In 240 of the 307 cases (78.2%), the skin cancer was found in strongly sun-exposed areas. A significant correlation between cyclin D1 expression and sun exposure was found only for dysplasia of SCC (Table 1). On the whole, however, the rate of positivity and average sum score of cyclin D1 of dysplasia were significantly higher in strongly sun-exposed areas (70.4%, 2.380) than in weakly sun-exposed areas (51.2%, 1.721; $P=0.0163$ and $P=0.0184$). In BOD, the rate of positivity and sum score of cancer nests were also significantly higher in the strongly sun-exposed than in the weakly sun-exposed

group (P=0.0223 and P=0.0182). In both SCC and BCC, which were not found to differ in sun exposure, the sum scores and rate of positivity of cyclin D1 expression in dysplasia were higher than those in BOD, as were the scores for sun exposure (Tables 1, 2).

Age and cyclin D1 expression

Overall, age was related to cyclin D1 score in regions of dysplasia (Table 1). In the individual diseases, however, this relationship was statistically significant only for BCC.

Growth and differentiation of cancer and cyclin D1 expression

As shown in Fig. 4, in well-differentiated SCC and in both strongly and weakly sun-exposed groups of BOD, a significant increase in the average sum scores (P<0.01) for cyclin D1 expression occurred not only in the first stage from normal skin to dysplasia, but also in the second stage, from dysplasia to cancer nests. However, in poorly and moderately differentiated subtypes of SCC and in adenoid and solid subtypes of BCC, this significant increase in average sum score occurred only in the first stage, but not in the second stage. There were no significant differences in scores among well-, moderately, and poorly differentiated SCC (P>0.1).

Discussion

Amplification and overexpression of cyclin D1 have been implicated in the pathogenesis of several types of human cancer, including breast [10, 23], esophagus [5, 14], liver [34], lung [28], bladder [16] and colorectum [1]. Cyclin D1 may not only provide prognostic information, but also be of diagnostic value in many cancers [6]. There have been excellent studies on the roles of cyclin D1 in skin cancer in some mouse models [21, 25–27, 33]. Even so, little is known concerning the roles of cyclin D1 in the carcinogenesis of human skin. Our study may be the first with a large sample of cyclin Dl expression in human nonmelanocytic skin cancer.

Exposure to UV irradiation from sunlight is believed to be the cause of the majority of human skin carcinomas. Bito et al. found no significant difference in cyclin D overexpression (using rabbit polyclonal anti-cyclin D) between UV-related (22 cases) and UV-unrelated SCCs (23 cases), though UV-related SCCs had a significantly higher incidence of p53 positivity [3]. In the present study, we used a large sample for analysis of nonmelanocytic skin cancer, and showed that the expression of cyclin D1 was significantly higher in dysplasia and in cancer nests in strongly sun-exposed areas than in those in weakly sun-exposed area, especially in the cases of SCC and BOD. Thus, our results confirmed the effect of sun exposure on the expression of cyclin D1. Further evidence is furnished by the observation that elimination of cyclin D1 resulted in enhanced sensitivity to radiation, resulting in a significant increase in apoptotic cells [15]. Adaptive survival response (ASR) in human cells, a phenomenon whereby the harmful effects of a high dose of exposure to ionizing radiation can be mitigated if cells are first exposed to a low dose(s) of radiation, correlated well with constitutively elevated levels of cyclin D1 [4]. Such cells demonstrate an increase in survival and a reduction in chromosomal aberrations, presumably due to the induction of DNA repair mechanism(s) [4]. Study of transfection of cyclin D1 provided further evidence that cyclin D1 plays a critical role in maintaining the integrity of the G_1/S checkpoint, via activation of apoptotic pathways following exposure to ionizing radiation in vitro [24]. As an early event, the elevated expression of cyclin D1 in dysplasia, which might be a result of sun exposure, is thought to influence the cellular response to radiation injury. Malignant tumor might be the result of failure of this response and/or the influence of other genes.

An interesting correlation was found in our study between patient age and cyclin D1 expression for dysplastic regions of BCC only, and not of SCC and BOD. Previous studies found no correlation between cyclin D1 expression and age in esophageal SCC [5], mammary infiltrating ductal carcinoma [8], Barrett's esophagus [2] or colorectal carcinogenesis [1]. Experiments in vitro, however, revealed that senescent human diploid fibroblasts expressed cyclin D1 at much higher levels than did young counterparts [9]. It was determined that increase in cyclin D1 expression in senescent cells is not due to arrested cell growth, but to specific cellular aging mechanisms [9]. The observed increase in cyclin D1 expression during cellular aging is due to an increase in binding activity of specific nuclear protein factors to an enhancer element, Sp1, and a decrease in binding to a silencer element in senescent cells [9]. In addition, the correlation between the expression of cyclin D1 and age for dysplastic regions of BCC may be related to greater cumulative sun exposure in older individuals, as mentioned above. According to the somatic mutation theory, aging phenotypes are the result of an accumulation of somatic mutations in the human body [19]. Wei et al. found that patients with reduced DNA repair capacities and overexposure to sunlight had an estimated risk of BCC over 5-fold that in a normal control group [32]. Cyclin D1 overexpression in cancer nests of BCC and the age-dependent cyclin D1 overexpression in dysplastic regions of BCC demonstrates that cyclin D1 may have a role as an aging-related early event in carcinogenesis of BCC.

Negative or only weak staining with anti-cyclin D1 antibody was observed in human normal tissues and benign tumors of skin and other organs [3, 10, 14, 26, 34]. In normal adult mouse skin, cyclin D1 immunostaining was not detected [27]. Compared with normal skin, higher levels of cyclin D1 expression were found in all tumors of mouse [33]. Therefore, cyclin D 1 was considered a useful marker of some malignant skin tumors [12]. In our study, although more than half of all cases

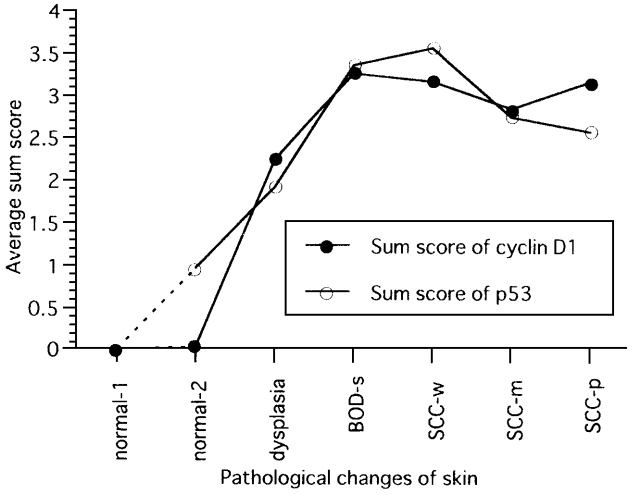


Fig. 5 Expressions of cyclin D1 and p53 in SCC and its precursors

were positive for cyclin D1 in cancer nests, only 2 of the 307 (<1%) cases exhibited positive staining in morphologically normal skin (Table 1). The normal skin in these 2 cases might have been undergoing malignant transformation manifesting morphological changes. Cyclin D1 appears to have different functions depending on the type of tumors. Overexpression of cyclin D1 in the mammary glands of transgenic mice leads to mammary hyperplasia and mammary adenocarcinoma formation [31], while cyclin D1-deficient mice exhibit defective mammary tissue proliferation [29]. Early appearance of cyclin D1 protein was reported in mouse skin carcinogenesis induced by a two-stage carcinogenesis protocol with 7, 12-dimethylbenz(a)anthracene (DMBA) / 12O-tetradecanoylphorbol-13-acetate (TPA) [25]. In contrast, cyclin D1 protein accumulation occurs in the majority of SCCs induced by complete carcinogenesis with benzo[α]pyrene (B[α]P), but is absent in early lesions [21]. Overexpression of cyclin D1 appeared to indicate a poor prognosis for SCC of the head and neck [20]. Cyclin D1 plays a role in progression to the invasive form of lobular carcinoma of the breast [23]. However, investigation of human materials to elucidate the roles of cyclin D1 in skin cancer is still incomplete. An interesting result of our study was the observation that dysplasia adjacent to cancer nests exhibited cyclin D1 expression similar to that of the cancer nests. In BOD and well-differentiated SCC, a significant increase in average sum scores occurred not only in the first stage from normal skin to dysplasia, but also in the second stage, from dysplasia to cancer nests. In poorly and moderately differentiated subtypes of SCC, however, this significant increase in average sum scores occurred only in the first stage, and not in the second (Fig. 4). There were also no significant differences in scores among well-, moderately and poorly differentiated SCC. These results suggest that in nonmelanocytic skin cancer, cyclin D1 expression is an early event occurring in the dysplastic stage. It has been commonly accepted that invasive SCC evolves from the precursor lesions of dysplasia and carcinoma in situ. On comparing sum scores of cyclin D1 as evolution order of invasive SCC from precursors including morphologically normal skin, dysplasia and strong sun-exposed group of BOD (Fig. 5), we found that after the peak value in strong sun-exposed BOD, the sharply ascending curve suddenly become flat with a declining tendency with more malignant differentiation of SCC. This finding suggests that in squamous cell malignancies (including BOD and SCC), overexpression of cyclin D1 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. A study of transgenic mice also provided in vivo evidence suggesting that the effect of cyclin D1 overexpression was restricted to proliferation and the mechanisms regulating the initiation of epidermal differentiation programs in these mice were not altered [26].

Cell-cycle regulation is a very complex phenomenon that requires the participation of a large number of gene families [33]. We compared the cyclin D1 expression observed in this study with the p53 expression observed in our previous study [17]. Our comparison showed that cyclin D1 expression was correlated with p53 expression mainly in dysplastic regions of BOD and well-differentiated SCC, especially in strongly sun-exposed group (data not shown). Fig. 5 presents a similar tendency of the cyclin D1 and p53 protein expressions during evolution of invasive SCC from the precursor lesions, except for the earlier appearance of p53 protein than cyclin D1 protein. In addition, rather like aberrant p53 expression [17], cyclin D1 overexpression also revealed some correlation with sun exposure and age. The relationship between cyclin D1 and p53 expression may hint at positive regulation of the expression of cyclin D1 by the aberrant p53 protein as an early event in carcinogenesis in the skin. Cyclin D1 expression has been considered to be regulated by p53 indirectly [11]. Wild-type p53 induces $p21^{Waf1/Cip1}$ expression, which can interact with several cyclins (including cyclin D1) and cyclin-dependent kinases and inhibit these kinases, inducing cell cycle arrest [7]. However, mutant p53 does not induce $p2l^{Waf1/Cip1}$ expression [7]. Therefore, as a result of p53 gene mutation, aberrant expression of p53 loss its function to induce $p2l^{Waf1/Cip1}$ expression and cell cycle arrest, removing the inhibition of cyclin D1 and speeding up the cell cycle clock. We postulate that aberrant p53 expression occurs prior to the overexpression of cyclin D1, and that in the early stage of carcinogenesis in skin the aberrant expression of p53 may elevate the overexpression of cyclin D1 to gain a growth advantage.

Overexpression of cyclin D1 is an early event in the processes of multistep carcinogenesis in skin cancer. During skin carcinogenesis, cyclin D1 overexpression might affect mainly the proliferation of tumor cells, but not their dedifferentiation. As an early event, the overexpression of cyclin D1 is related to sun exposure in carcinogenesis of SCC and BOD. In carcinogenesis of BCC,

however, cyclin D1 expression is an aging-dependent early process. Prior aberrant p53 expression in some skin cancers might regulate the expression of cyclin D1 to gain growth advantage in carcinogenesis of skin. These findings suggest that the overexpression of cyclin D1 has important and different roles in BOD, SCC and BCC.

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