Research Article for Cancer Science

Alteration of p53-binding protein 1 expression during skin carcinogenesis: association with genomic instability

Short title: 53BP1 and genomic instability in skin cancer

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Brief statements: Defective DNA damage response (DDR) can result in genomic instability (GIN) which is considered to be a central aspect of any carcinogenic process. P53-binding protein 1 (53BP1) belongs to a family of evolutionarily conserved DDR proteins. This study demonstrated a number of nuclear 53BP1 foci in human skin tumorigenesis, suggesting a constitutive activation of DDR in skin cancer cells. The results of this study suggest that GIN has a crucial role in the progression of skin carcinogenesis.

Summary

Epidermal cells are the first cells to be exposed to environmental genotoxic agents such as ultraviolet (UV) and ionizing radiations (IR), which induce DNA double strand breaks (DSBs) and activate DNA damage response (DDR) to maintain genomic integrity. Defective DDR can result in genomic instability (GIN) which is considered to be a central aspect of any carcinogenic process. P53-binding protein 1 (53BP1) belongs to a family of evolutionarily conserved DDR proteins. Because 53BP1 molecules localize at the sites of DSBs and rapidly form nuclear foci, the presence of 53BP1 nuclear foci can be considered as a cytological marker for endogenous DSBs reflecting GIN. The levels of GIN were analyzed by immunofluorescence studies of 53BP1 in 56 skin tumors that included 20 seborrheic keratosis (SK), 8 actinic keratosis (AK), 9 Bowen's disease (BD), 9 squamous cell carcinoma (SCC), and 10 basal cell carcinoma (BCC). This study demonstrated a number of nuclear 53BP1 foci in human skin tumorigenesis, suggesting a constitutive activation of DDR in skin cancer cells. Because AK showed a high DDR type of 53BP1 immunoreactivity, GIN seems to be induced at the precancerous stage. Furthermore, invasive cancers exhibited a high level of intense, abnormal 53BP1 nuclear staining with nuclear accumulation of p53, suggesting a disruption of DDR leading to a high level of GIN in cancer cells. The results of this study suggest that GIN has a crucial role in the progression of skin carcinogenesis. The detection of 53BP1 expression by immunofluorescence can be a useful histological marker to estimate the malignant potential of human skin tumors.

Introduction

The skin is the primary barrier for humans against the external environment. Therefore, epidermal cells are the first cells to be exposed to physical and chemical genotoxic agents such as ultraviolet (UV) and ionizing radiations (IR). IR effectively induces DNA double strand breaks (DSBs) in normal cells and activates DNA damage response (DDR) pathways to maintain genomic integrity. DDR genes, such as p53, are frequently mutated in human cancer. Thus, defective DDR can result in genomic instability (GIN) which is generally considered to be a central aspect of any carcinogenic process.[1,2] It has been shown that gamma irradiation induced skin tumors in mice. Most of the tumor-bearing mice showed a loss of the wild-type p53 allele. Since no skin tumor was found in wild-type p53 mice, this suggested a requirement of p53 loss in irradiation-induced skin carcinogenesis.[3] The incidence of skin cancer was reported to be elevated in atomic bomb survivors, which also suggested a radiation etiology in human skin carcinogenesis.[4]

P53-binding protein 1 (53BP1) belongs to a family of evolutionarily conserved DDR proteins with C-terminal BRCT domains.[5, 6] The 53BP1 is a nuclear protein that rapidly localizes at the sites of DSBs, and co-operatively activates p53 with other kinases.[7-12] Subsequently, activated p53 plays a critical role in cellular responses to genomic injury, such as cell cycle arrest, DNA repair, and apoptosis.[13, 14] It has been well documented *in vitro* with immunofluorescence that 53BP1 exhibits diffuse nuclear staining in untreated primary cells. However, after exposure to IR, 53BP1 localizes at the sites of DSBs and forms discrete nuclear foci.[7,8,15,16] We have recently demonstrated that immunofluorescence analysis for 53BP1 specifically detected the 53BP1 nuclear foci at

the sites of DSBs induced by IR in formalin-fixed paraffin-embedded mouse intestine.[17] Because one manifestation of GIN is the induction of endogenous DSBs,[18] the level of 53BP1-focus formation can be considered as a cytological marker for GIN.

Human cancers develop through a multistep process that involves the accumulation of genetic mutations.[19] It is well established that any DNA damages can lead to GIN and subsequently induce DDR. Thus, measurement of GIN, a hallmark feature of solid tumors that is implicated in both initiation and progression of cancers, may serve as a valuable molecular marker of malignant potential. Our recent study of thyroid tumors from patients demonstrated a few nuclear 53BP1 foci in follicular adenoma but conspicuously more nuclear 53BP1 foci in thyroid cancers. This suggested a constitutive activation of DDR in thyroid tumors and increased GIN with progression of cancer.[17] Furthermore, anaplastic thyroid cancers prominently exhibited an abnormal and intense nuclear staining of 53BP1, which was also observed in mouse colonic crypts as a delayed response to a lethally high dose of IR, suggesting increased GIN with progression to high-grade cancer.[17] Thus, we propose that immunofluorescence analysis of 53BP1 expression can be a useful tool to estimate the level of GIN and, simultaneously, the malignant potential of human thyroid tumors. The present study analyzed the presence of GIN by immunofluorescence of 53BP1 expression in a series of skin tissues from patients to evaluate the significance of GIN during skin carcinogenesis. Similar to thyroid tumorigenesis, GIN was shown to be induced in skin cells at a precancerous stage and increased significantly with progression to cancer.

Subjects and Methods

Skin tumor tissues

A total of 56 archival skin tissue samples, which were obtained from surgically excised specimens, were selected for this study from the archives of our department (Table 1). Histologically, the 56 primary skin tumors were comprised of the following: 20 seborrheic keratosis (SK) arising from 10 sun-exposed (including 7 faces and 3 necks) and 10 non-exposed sites (including 4 abdomens, 3 chests, 2 axillae, and 1 backs); 8 actinic keratosis (AK); 9 Bowen's disease (BD); 9 squamous cell carcinoma (SCC); and 10 basal cell carcinoma (BCC). All samples were formalin-fixed paraffin-embedded tissues, from which sections were prepared for immunofluorescence studies. For normal controls, 14 samples of non-exposed (including 5 abdomens, 5 backs, 2 ramps, 1 chest, and 1 axilla) and 8 samples of sun-exposed (including 5 faces, 1 neck, 1 hand, and 1 leg) normal epidermal cells surrounding tumor sections were evaluated.

Immunofluorescence

After antigen retrieval with microwave treatment in citrate buffer, deparaffinized sections were preincubated with 10% normal goat serum. Tissue sections were then reacted with anti-53BP1 rabbit polyclonal antibody (Bethyl Labs, Montgomery, TX, USA) at a 1:200 dilution. The slides were subsequently incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). Specimens were counterstained with 4', 6-diamidino-2- phenylindole dihydrochloride (DAPI-I,

Vysis Inc.), and were visualized and photographed using a fluorescence microscope (Zeiss Axioplan2, Carl Zeiss Japan, Tokyo, Japan) equipped with a CCD camera, and then analyzed with IPLab/MAC image software (Scanalytics Inc., Fairfax, VA). Signals were analyzed in 10 viewing areas per case at a 1,000-fold magnification.

Evaluation of immunofluorescence results

As described in our previous report,[17] the pattern of 53BP1 immunoreactivity was classified into four types: 1) Stable type: Faint and diffuse nuclear staining, 2) Low DDR type: One or two discrete nuclear foci, 3) High DDR type: Three or more discrete nuclear foci, and 4) Abnormal type: Intense heterogeneous nuclear staining, occasionally, with several small foci. The percentage of epidermal or tumor cells expressing each type of 53BP1 immunoreactivity in each viewing area was graded into the following four groups: 1) Negative: 0 to less than 5%, 2) Low: 5% to less than 30%, 3) Medium: 30% to 60%, and 4) High: More than 60%. The type of 53BP1 expression pattern in each case was determined by the predominant expression pattern.

Statistical analysis

The Mann-Whitney test was used to assess differences in the type of 53BP1 expression between non-exposed and exposed epidermal cells. Spearman's correlation coefficient by rank test was used to assess correlation between histological type of skin tumors and type of 53BP1 expression. A p-value of less than 0.05 was considered statistically significant.

Double-labeled immunofluorescence

To assess the co-localization of 53BP1-foci formation and p53 expression, double-labeled immunofluorescence was performed. We also carried out a double-labeled immunofluorescence of 53BP1 and Ki-67 expression to clarify the association of type of 53BP1 expression and cycling tumor cells. In double staining, tissues were incubated with a mixture of rabbit anti-53BP1 antibody and mouse anti-p53 monoclonal antibody (DO7, DAKO) at a 1:200 dilution or mouse anti-Ki-67 monoclonal antibody (MIB-1, DAKO) at a 1:50 dilution, and subsequently incubated with a mixture of Alexa Fluor 488-conjugated goat anti-rabbit antibody and Alexa Fluor 546-conjugated goat anti-mouse antibody. Specimens were counterstained with DAPI-I (Vysis Inc.), and were visualized and photographed using a fluorescence microscope (Zeiss Axioplan2) equipped with a CCD camera, and then analyzed with IPLab/MAC image software (Scanalytics Inc.). Signals were analyzed at a 1,000-fold magnification.

Results

53BP1 expression in normal epidermis surrounding tumors

Of the 14 controls consisting of non-exposed normal epidermis, 13 cases (92.9%) expressed only stable type cells (Figure 1A), while one case (7.1%) showed stable type keratinocytes but also included a small number (up to 10%) of low DDR type in the basal layer. Of the other 8 controls consisting of sun-exposed normal epidermis, 2 cases (20%) expressed only stable type cells, while 5 cases (62.5%) showed stable type in more than 70% of keratinocytes but also had up to 30% of low DDR type (Figure 1B) in the basal layer. One other case (12.5%) also showed stable type in more than 70% of keratinocytes, but included up to 30% of high DDR type in the basal layer.

53BP1 expression in skin tumors

The results of the immunofluorescence of staining patterns for 53BP1 in each histological type of skin tumors are presented in Table 2. Similar to the pattern observed for normal epidermal cells, all 10 of the non-exposed SK cases expressed only the stable type (Figure 2A). Of the 10 sun-exposed SK cases, 4 (40.0%) expressed only the stable type, while 6 (60.0%) showed stable type in more than 70% of tumor cells but also up to 30% of low DDR type (Figure 2B) mainly in basal layer.

In contrast to the normal epidermis and SK, of the 8 AK cases, 4 cases (50.0%) and 3 cases (37.5%) showed high DDR (Figure 2C) and low DDR types respectively in dysplastic cells, while only one case (12.5%) expressed the stable type. In the 9 BD cases, 2 cases (22.2%) showed low DDR type, 4 cases (44.4%) were of high DDR type,

and 2 cases (22.2%) were of mixed high DDR and abnormal type (Figure 2D), while only one case (11.1%) expressed the stable type. Of the 9 SCC cases, 4 (44.4%) were of mixed low DDR and abnormal type and 5 (55.6%) were of the abnormal type (Figure 2E). Finally, of the 10 BCC cases, one case (10.0%) was of mixed low DDR and abnormal type, and 9 cases (90.0%) were of the abnormal type (Figure 2F).

The Mann-Whitney test revealed that DDR expression of 53BP1 was significantly higher in the sun-exposed epidermis than in the non-exposed epidermis (P=0.001). Furthermore, Spearman's analysis revealed that the histological type of skin tumors was significantly correlated with type of 53BP1 expression (P<0.001).

Double-labeled immunofluorescence of 53BP1 and p53 expressions (**Figure 3**)*/Ki-67 expressions* (**Figure 4**)

Normal epidermis and SK expressed the stable or a low DDR type of 53BP1 immunoreactivity and only a few Ki-67 nuclear staining at the basal layer but no p53 nuclear staining. Discrete nuclear foci in a high DDR type of 53BP1 immunoreactivity were observed and were co-localized to dysplastic cells exhibiting p53 nuclear staining at the basal layer in AK. In BD, p53 nuclear staining was sparsely found in cancer cells which were distributed throughout the epidermal layer, and discrete nuclear foci of high DDR type of 53BP1 immunostaining were co-localized to p53-positive cancer cells. In SCC and BCC, high levels of abnormal type of 53BP1 and strong p53 immunoreactivity were observed in nuclei of cancer cells, and cells expressing the abnormal type of 53BP1 immunoreactivity were randomly distributed in lesions. Intense 53BP1 staining was not always co-localized with p53 overexpression in cancer cells. Furthermore, double staining of 53BP1 and Ki-67 demonstrated that discrete nuclear foci of 53BP1

immunostaining were not co-localized to Ki-67-positive dysplastic/cancer cells in AK/BD; whereas abnormal type of 53BP1 staining frequently expressed Ki-67 nuclear staining.

Discussion

Development of SCC of the skin is viewed as a multistep process, while BCCs are believed to develop *de novo*.[20] In skin carcinogenesis, AK is a well established precancerous skin lesion and it has been suggested that ~10% of these sun-induced lesions will develop into SCC.[21] BD, also known as carcinoma *in situ* (CIS), represents a preinvasive stage of SCC. The present study demonstrated apparent differences in 53BP1 staining patterns during human skin tumorigenesis, as in the following: SK/benign tumor, AK/precancerous lesion, BD/CIS, and SCC or BCC/invasive cancer. The number of discrete immunoreactive nuclear foci in DDR type of 53BP1 in the epidermis seems to increase in precancerous lesions. Furthermore, the abnormal type of 53BP1 immunoreactivity was restricted to malignancies including both CIS and invasive cancers. Similar results, which showed the differences in 53BP1 expression patterns during carcinogenesis, were also obtained in our recent study on thyroid tumors resected from patients.[17] Therefore, we submit that immunofluorescence analysis of 53BP1 expression can be a useful tool to estimate the level of GIN and, simultaneously, the malignant potential of human tumors such as skin and thyroid tumors.

Interestingly, the low DDR type of 53BP1 immunoreactivity was very rare in the non-exposed epidermis but frequently found in sun-exposed skin. Similarly, sun-exposed SK/benign skin tumor also frequently showed a low DDR type of 53BP1 immunoreactivity, but none of the non-exposed SK cases expressed the DDR type of 53BP1 immunoreactivity. Because the skin is the primary barrier in humans against the external environment, sun-exposed epidermis is continuously exposed to a low level of physical and chemical genotoxic agents such as UV and IR. Thus, a low DDR type of

53BP1 immunoreactivity in the sun-exposed epidermis may represent a minor genotoxic injury induced by external environmental factors.

P53 is activated by DDR-associated molecules, and is essential to control GIN and to suppress tumorigenesis.[22] The p53 mutation is the most prominent aberration in skin cancers, and it is now established that ~50% of all skin cancers show p53 mutations.[23] Double-labeled immunofluorescence was carried out to evaluate the association between the type of 53BP1 expression and p53 nuclear staining. In this study, although no p53 staining was observed in the epidermis and SK even at the sun-exposed sites which showed a low DDR type of 53BP1 immunoreactivity, AK showed both p53 nuclear staining and a high DDR type of 53BP1 immunoreactivity in dysplastic cells at the basal layer in the epidermis. The co-localization of p53 nuclear staining and 53BP 1 nuclear foci was also observed in BD. P53 mutations have been found in precanceous epidermis[24-26] and can be detected by immunohistochemistry. Indeed, 70% of immunohistochemical detections were shown to have an underlying mutation in the p53 gene.[25, 27] Because of the loss of p53 function based on gene mutation, environmental genotoxic agents may easily induce DSBs in precancerous lesion/CIS in skin. Alternatively, because this study also demonstrated no co-localization of DDR type 53BP1 expression and Ki-67 nuclear staining as a marker for cycling cells in AK and BD, activated DDR induced by genotoxic factors may activate p53 function to induce cell cycle arrest in injured cells. Furthermore, a high level of the abnormal type of 53BP1 immunoreactivity was restricted to invasive cancer and was significantly associated with p53 overexpression reflecting mutation. However, cancer cells exhibiting an abnormal type of 53BP1 staining were co-localized with Ki-67 staining but not always co-localized with p53 staining. Thus, cancer cells expressing strong p53

immunoreactivity was cycling cells, suggesting a loss of p53 function and a disruption of the DDR pathway; whereas AK and BD cells exhibiting both 53BP1 nuclear foci and weak p53 staining was non-cycling cells, suggesting a p53 function through activated DDR pathway. Taken together, these findings may indicate that GIN may have already occurred at the precancerous stage during skin tumorigenesis, and that a loss of control of GIN based on p53 mutations may allow further accumulation of other genomic alterations to progress to invasive cancer through acceleration of cell proliferating.

In summary, this study demonstrated a number of nuclear 53BP1 foci in skin tumors resected from patients, which were similar to those found in irradiated cells, suggesting a constitutive activation of DDR in skin cancer cells. Because AK showed a high DDR type of 53BP1 immunoreactivity which co-localized with p53 nuclear staining as well as BD, GIN seems to be induced at the precancerous stage. Furthermore, invasive cancers exhibited the abnormal type of 53BP1 immunoreactivity with an underlying mutation in the p53 gene and an increased proliferation, suggesting disrupted DDR subsequently leading a high level of GIN in cancer cells. This study proposed that GIN has a crucial role in the progression of skin carcinogenesis. Immunofluorescence analysis of 53BP1 expression of various tumor tissues should be performed to clarify the significance of 53BP1 staining pattern as a common histological marker to estimate the malignant potential of human tumors.

Acknowledgments

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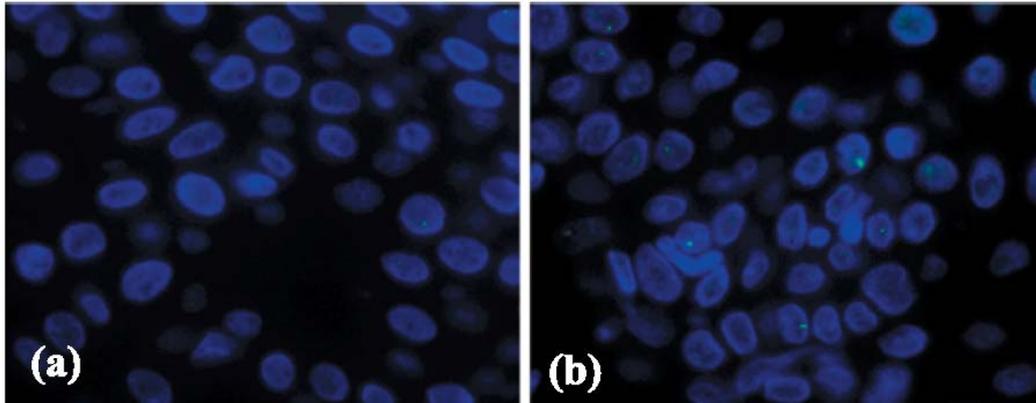
Figure Legends

Figure 1. Immunofluorescence of 53BP1 expression in normal epidermis surrounding tumor sections. Non-exposed epidermis showed a stable type staining and rarely one nuclear focus (A). Sun-exposed epidermis occasionally showed one discrete 53BP1 nuclear focus at the basal layer (B).
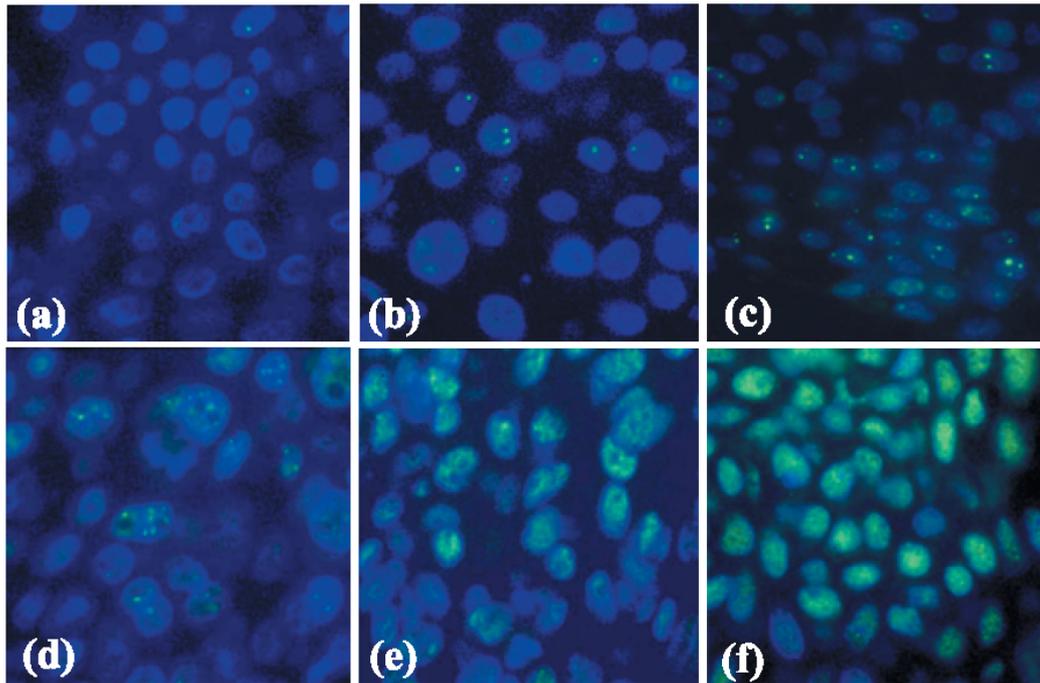
Figure 2. Immunofluorescence of 53BP1 expression in human skin tumors. Seborrheic keratosis (SK) in the non-exposed skin expressed stable type staining with rarely one nuclear focus (stable type) (A), whereas SK in the sun-exposed skin expressed an occasional one or two nuclear foci [low DNA damage response (DRR) type] (B). Actinic keratosis showed three or more discrete nuclear foci in dysplastic cells (high DRR type) (C). Bowen's disease showed several discrete nuclear foci mixed with intense and heterogeneous nuclear staining (mixed DRR and abnormal type) (D). Squamous cell carcinoma (E) as well as basal cell carcinoma (F) exhibited intense and heterogeneous nuclear staining (abnormal type).
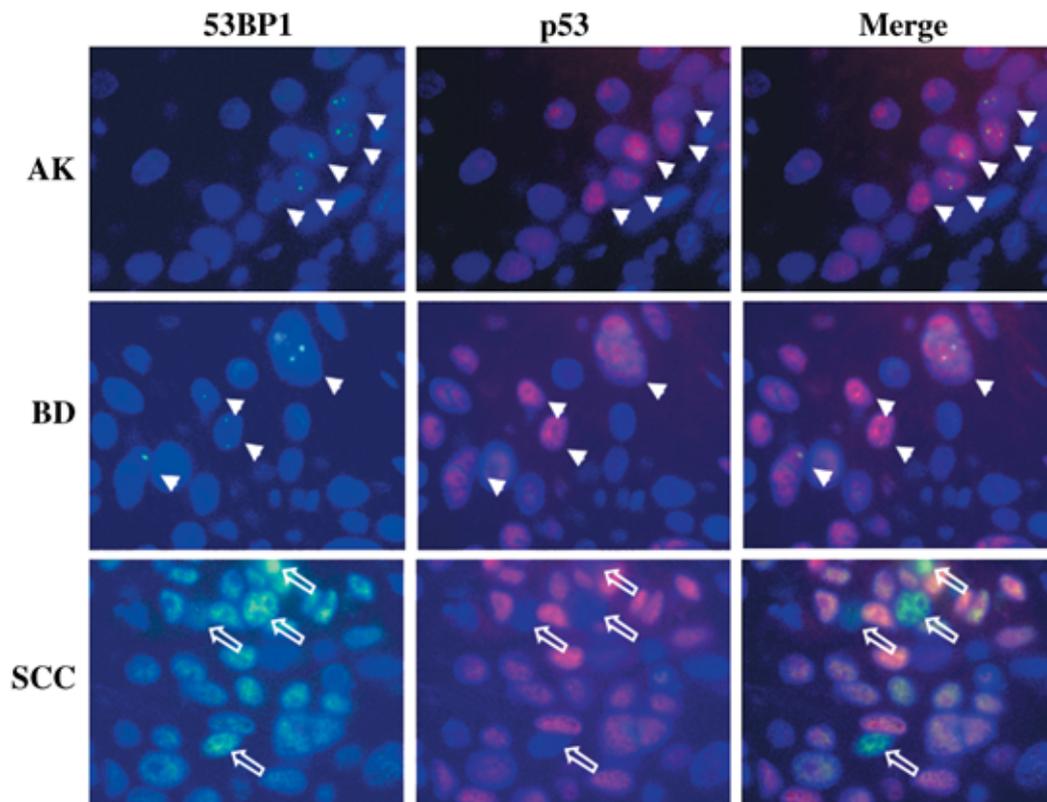
Figure 3. Double-labeled immunofluorescence for 53BP1 and p53 expression. Actinic keratosis (AK) showed co-localization of discrete 53BP1 nuclear foci and p53 nuclear staining in dysplastic cells at the basal layer, suggesting an activation of DNA damage response (DDR). Bowen's disease (BD) also showed co-localization of discrete 53BP1 nuclear foci and p53 nuclear staining in dysplastic cells including dispersed plump cells. Squamous cell carcinoma (SCC) exhibited intense and heterogeneous nuclear staining of both 53BP1 and p53 immunoreactivity, however, intense 53BP1 staining was not always co-localized with p53 overexpression, suggesting a disruption of the DDR pathway. Arrows indicate co-localization of 53BP1 nuclear foci and p53 staining in both AK and BD. Open arrows indicate cancer cells showing intense 53BP1 staining with no p53 staining in SCC.
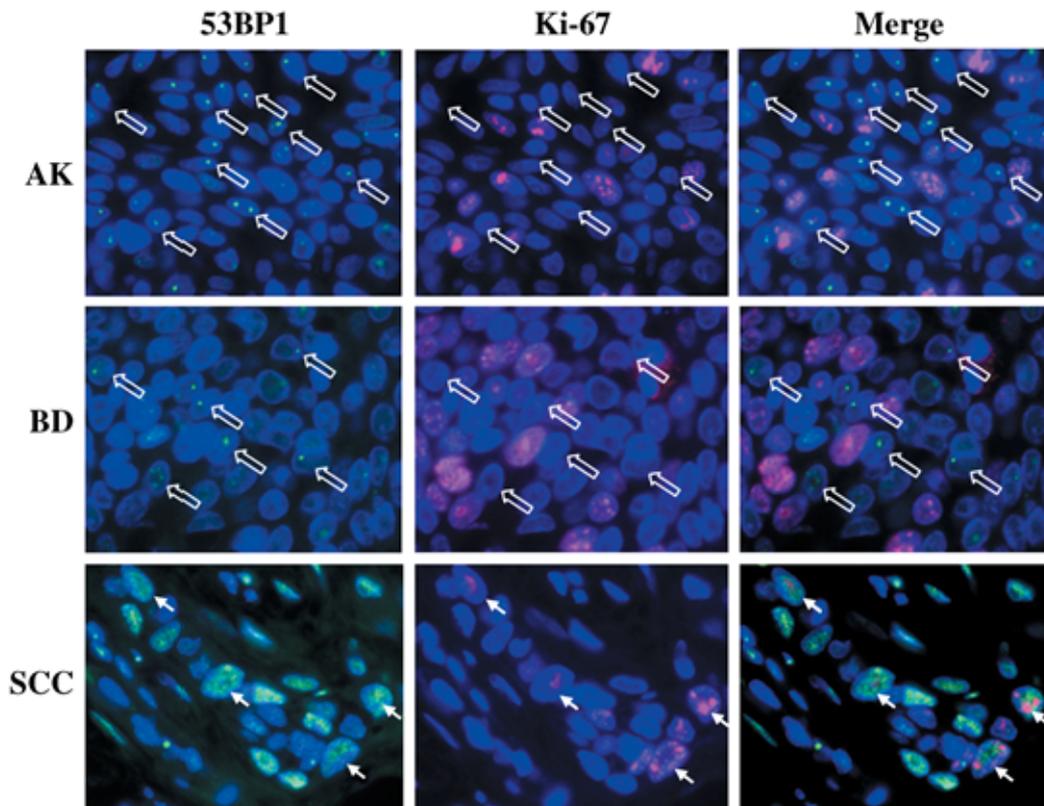
Figure 4. Double-labeled immunofluorescence for 53BP1 and Ki-67 expression. Actinic keratosis (AK) showed no co-localization of discrete 53BP1 nuclear foci and Ki-67 nuclear staining at the basal layer. Bowen's disease (BD) also showed independent discrete 53BP1 nuclear foci from Ki-67 nuclear staining in cancer cells. Squamous cell carcinoma (SCC) occasionally exhibited intense and heterogeneous nuclear staining of both 53BP1 and Ki-67 immunoreactivity, suggesting a proliferating ability through a disruption of the DDR pathway. Open arrows indicate cells showing 53BP1 nuclear foci with no Ki-67 staining in both AK and BD. Arrows indicate co-localization of intense 53BP1 staining and Ki-67 staining in SCC.
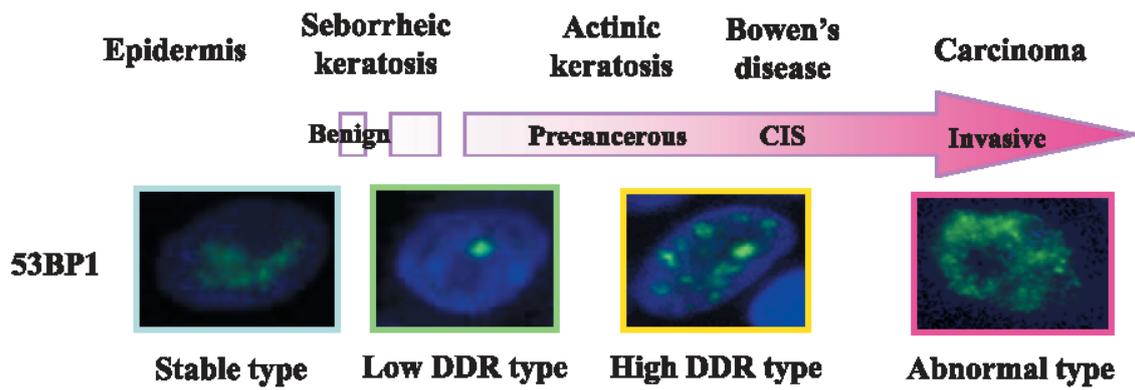
Figure 5. Schematic presentation showing differences in 53BP1 staining patterns during skin tumorigenesis.

DDR: DNA damage response, CIS: carcinoma *in situ*

Table-supplement. Odds ratio (OR) and 95% confidence interval (CI) for the incidence of DNA damage response expression of 53BP1 in both normal epidermis and seborrheic keratosis samples

	Normal epidermis			Seborrheic keratosis		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
ATD	1.09	0.98-1.21	0.126	1.00	0.90-1.12	0.977
Sex*	2.26	0.22-22.9	0.492	29.9	2.16-414	0.011

*Female vs. Male

ATD: age at the time of diagnosis

Table 1. Summary of subjects used in this study

Histological type	n	Mean age (range)	M/F	Site (non-exposed/ sun-exposed)
Normal epidermis				
non-exposed	14	60.8 (31-80)	11/3	14
sun-exposed	8	79.9 (66-88)	3/5	8
Seborrheic keratosis				
non-exposed	10	65.6 (58 – 90)	10 / 0	10
sun-exposed	10	77.9 (53 – 91)	3 / 7	10
Actinic keratosis	8	79.5 (66 – 91)	3 / 5	0/8
Bowen's disease	9	76.5 (66 – 88)	3 / 6	1/8
Squamous cell carcinoma	9	82.3 (74 – 96)	3 / 6	0/8
Basal cell carcinoma	10	73.3 (53 – 89)	5 / 5	3/7

Table 2. Results for type of 53BP1 expression in skin tumors by immunofluorescence

	n	Stable	Low DDR	High DDR	Mixed DDR and abnormal	Abnormal
Epidermis						
non-exposed	14	13 (92.9%)	1 (7.1%)	0	0	0
sun-exposed	8	2 (25.0%)	5 (62.5%)	1 (12.5%)	0	0
SK						
non-exposed	10	10	0	0	0	0
sun-exposed	10	4 (40.0%)	6 (60.0%)	0	0	0
AK	8	1 (12.5%)	3 (37.5%)	4 (50.0%)	0	0
BD	9	1 (11.1%)	2 (22.2%)	4 (44.4%)	2 (22.2%)	0
SCC	9	0	0	0	4 (44.4%)	5 (55.6%)
BCC	10	0	0	0	1 (10.0%)	9 (90.0%)

DDR: DNA damage response

SK: seborrheic keratosis

AK: actinic keratosi

BD: Bowen's disease

SCC: squamous cell carcinoma

BCC: basal cell carcinoma