Reproducibility and Expression of Skin Biomarkers in Sun-Damaged Skin and Actinic Keratoses

Janine G. Einspahr,[1] Min-Jian Xu,[1] James Warneke,[3] Kathylynn Saboda,[4] James Ranger-Moore,[4] Paul Bozzo,[2] Laura Duckett,[4] Rayna Goldman,[4] Po Lin,[4] Julie Buckmeier,[4] and David S. Alberts[1]

Departments of [1]Medicine, [2]Pathology, [3]Surgery, and [4]Arizona Cancer Center College of Medicine, Mel and Enid Zuckerman, College of Public Health, University of Arizona, Tucson, Arizona

Abstract

Objectives: **To explore p53 and proliferating cell nuclear antigen (PCNA) expression and polyamine content as biomarkers in skin cancer chemoprevention trials, we evaluated their expression in early stages of UV-induced squamous cell tumorigenesis.**
Methods: **Biopsies were collected from three groups: 78 subjects with sun damage on forearms, 33 with actinic keratosis (AK) on forearms, and 32 with previous squamous cell carcinoma. Participants with sun damage were randomized to sunscreen or no sunscreen.**
Results: **We found significant differences in p53 and polyamines in forearms from the sun-damaged group (11.5 ± 1.2% for p53, 65.5 ± 1.9 nmol/g for putrescine, and 187.7 ± 3.3 nmol/g for spermidine) compared with the group with sun damage plus AK (20.9 ± 2.3% for p53, P = 0.0001; 81.7 ± 3.9 nmol/g for putrescine, P = 0.0001; 209.4 ± 8.2 nmol/g for spermidine, P < 0.06). PCNA was not different. When lesion histology was considered, there was a stepwise significant increase in p53 in biopsies without characteristics of AK compared with early AK (P = 0.02) and AK (P = 0.0006) and a similar pattern for PCNA with the only significant difference between early AK and AK. There was a stepwise increase in putrescine and spermidine in normal, sun-damaged forearm, forearm from subjects with AK, and the AK lesion itself (P < 0.0001). No significant differences in p53 or polyamines were seen in 3-month biopsies or, as a result of sunscreen use, although PCNA in the sun-damaged group not using sunscreen decreased significantly.**
Conclusions: **p53 expression and polyamines in skin were elevated in early stages of skin tumorigenesis and were not affected by sunscreen, adding validity to their use as biomarkers in skin cancer chemoprevention trials.** (Cancer Epidemiol Biomarkers Prev 2006;15(10):1841–8)

Background

Nonmelanoma skin cancers, squamous cell carcinoma (SCC), and basal cell carcinoma are the most common human cancers with over 1 million cases estimated in 2005 (1). The majority of nonmelanoma skin cancers are due to chronic exposure to UV light with over 80% of these cancers found on sun-exposed areas of the body (2). The incidence rates for nonmelanoma skin cancer correspond well with increased UV exposure as shown by the increased incidence among individuals with occupational or recreational outdoor exposure or who reside at latitudes closer to the equator (2).

There is strong evidence that actinic keratoses (AK) are precursors of SCC. The most important risk factors for both AK and SCC are a genetic propensity or ''fair skin phenotype'' and cumulative sun exposure. Although the epidemiology of SCC and AK are nearly identical, SCCs tend to occur most often on the head, whereas AKs are primarily located on the upper extremities. Moreover, AK are an important risk factor for identifying those at increased risk of SCC (3). Although the exact incidence of AK is unknown, they are far more prevalent than SCC (3-5). Moreover, ~60% of SCCs arise from a preexisting AK (6). Although most AK do not progress to SCC, AK represents SCC *in situ* at its earliest stages (7). Skin adjacent to AK often contain similar histologic changes, suggesting that AK develop on a background of sun-damaged skin (6). These findings support the concept that there is a field of cancerization due to chronic exposure to UV light (6).

Mutations of the *p53* gene occur early in the sequential development of chronically sun-damaged skin, AK, and SCC (8-14), strongly supporting an important role for p53 in UV-induced skin carcinogenesis. The *p53* gene is essential in maintaining genomic integrity by blocking DNA replication in response to DNA damage from exposure to agents like UV light. Cells with extensive damage are blocked from entering the cell cycle and instead undergo apoptosis (15, 16).

Biomarkers for chemoprevention studies must be reliable, sensitive and specific, quantitative, measurable in reasonably accessible biological samples, on or closely related to the causal pathway, and capable of modulation by chemoprevention agents (17). Biomarkers can be broadly categorized as markers with biological relevance to the carcinogenic pathway, such as intraepithelial neoplasias or measures of proliferation [i.e., proliferating cell nuclear antigen (PCNA)] or apoptosis; or as markers that show the effect of a particular agent. This is exemplified by the suppression of polyamine levels in tissue treated with the polyamine synthesis inhibitor, difluoromethylornithine (18). Although clinical end points, such as tumor incidence and number, are obvious targets for chemoprevention studies, the addition of measures that reflect basic cellular events (histologic change, cell proliferation) and molecular targets (nuclear retinoid receptors, p53 mutation/overexpression) or pathways (polyamine synthesis) are also good biomarker candidates. These end points could function as biomarkers in chemoprevention studies and aid in determining the mechanism(s) by which an agent can act to alter the carcinogenesis process.

The goal of the current study was to determine whether PCNA and p53 expression and polyamine content are differentially expressed in the early stages of UV-induced skin carcinogenesis and whether these biomarkers are stable at a 3-month time point compared with baseline.

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Requests for reprints: Janine G. Einspahr, Arizona Cancer Center, PO Box 245024, Tucson, AZ 85724. Phone: 520-626-2444; Fax: 520-626-9275. E-mail: jeinspahr@azcc.arizona.edu

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Materials and Methods

Study Population. Subjects were recruited in a number of ways that included contacting subjects who participated in earlier skin chemoprevention trials, distribution of flyers, and media advertising (i.e., television, radio, newspapers). Males and females ages ≥18 years that were willing to limit sun exposure were recruited for the study. Exclusion criteria included immunosuppression, serious concurrent illness, invasive cancer, or cancer treatment within the past 5 years, and baseline laboratory values outside of normal limits. The study was approved by the Institutional Review Board review of the University of Arizona.

Three groups of participants were recruited: Group 1 had clinical evidence of sun damage (i.e., at least mild fine wrinkling, coarse wrinkling, mottling, and hyperpigmentation) on posterior forearms but no clinical evidence of frank AK and no prior history of skin cancer (91 subjects were accrued, 2 were not randomized, and 78 completed the study). Group 2 had at least five clinically visible AK lesions on posterior forearms and no prior history of skin cancer (38 subjects were accrued and 33 completed the study). Group 3 had pathology-verified SCCs removed within the previous 12 months of the study (35 subjects were accrued and 23 completed the study).

As shown in Fig. 1, the study schema, there was a 2-week run-in during which participants applied study-provided sunscreen (SPF 50) daily after which baseline biopsies were done. Participants in group 1 were randomized to either no sunscreen (n = 45) or daily sunscreen (n = 43) for 3 months while all other groups applied sunscreen daily for the 3-month period. Biopsies were repeated at the end of 3 months. All three groups underwent three 4-mm punch biopsies taken from posterior forearms at baseline and after 3 months. The baseline and 3-month biopsies were from adjacent sites on the forearm so that a similar area of the arm was sampled. Group 2 (clinical AK) had forearm biopsies done from an area that did not include a clinical AK and in addition had 4-mm punch biopsies from two different clinical AK from the posterior forearms removed at baseline and at 3 months. Group 3 (previous SCC) had 4-mm biopsies removed from sites adjacent to the previously resected SCC and also had matching forearm biopsies done at baseline and at 3 months. One biopsy from all forearms was fixed in 10% neutral buffered formalin for p53 and PCNA expression, one biopsy was frozen for future use, and one biopsy was delivered to the laboratory on ice for polyamines. Group 2 biopsies from the AK and group 3 biopsies from sites adjacent to the previous SCC resection site were fixed (for p53 and PCNA) and frozen only, polyamine analyses were not done on these samples.

To determine whether polyamine levels differed in normal skin compared with sun-damaged skin and AK, additional sample sets of normal skin samples and AK were collected. Punch biopsies (4 mm) were obtained from AK lesions in 33 subjects who had at least five discrete, clinically diagnosed AKs on sun-exposed surfaces of each forearm. Normal-appearing skin samples were obtained from 34 healthy individuals (20 from normal-appearing skin samples obtained during face-lift surgical procedures and 14 from normal-appearing upper inner arm skin).

Histopathology of AK. Formalin-fixed, paraffin-embedded biopsies were stained by routine H&E. The dermatopathologist graded early AK based on the following criteria: minimal cytologic atypia in basilar and sometimes suprabasilar layers

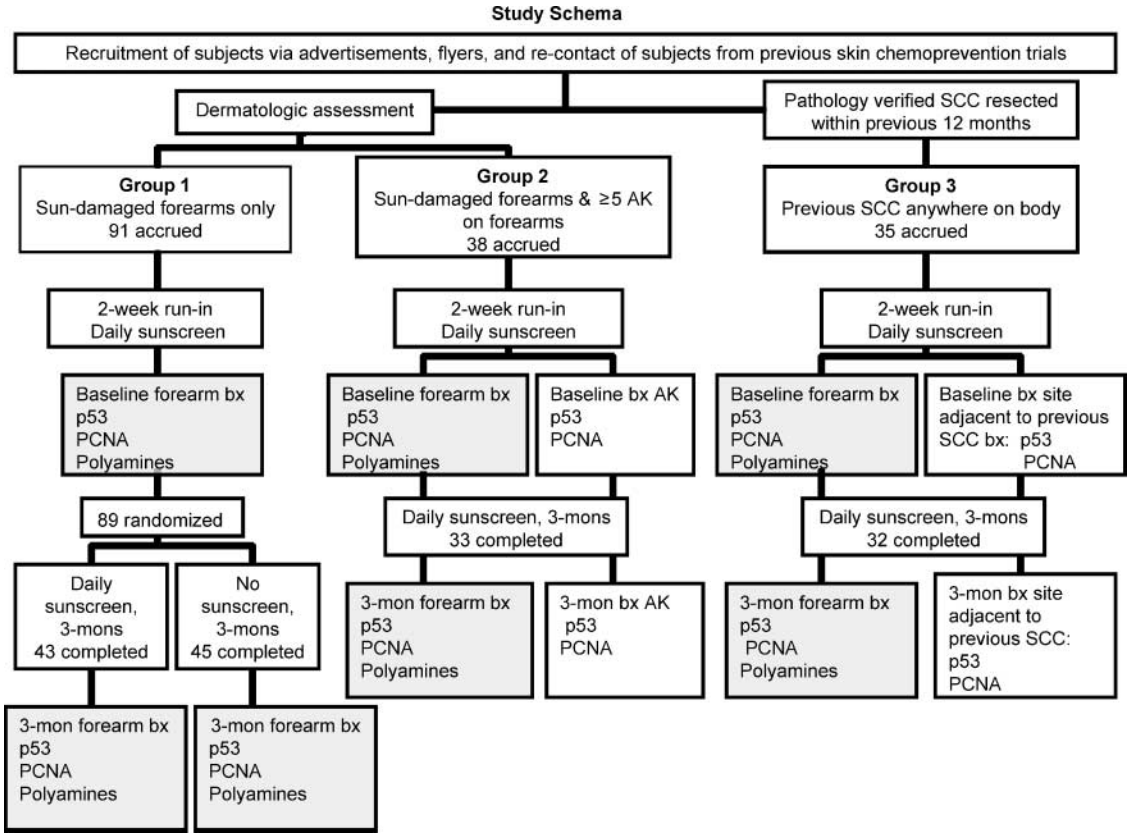

Figure 1. Study schema for the three groups of subjects in the biomarkers trial. Group 1 had sun damage on forearms but no evidence of AK, group 2 had sun damage and AK on forearms, and group 3 had an SCC resected within the 12 months before the trial. The subset of samples collected from normal upper inner arm and facelift skin and AK lesions for polyamine analysis are not shown.

in a limited number of cells. Criteria for AK included prominent basal layer atypia and/or suprabasilar atypia involving more than a few cells but not full thickness involvement of Bowen's (SCC *in situ*) or follicular sparing. A category termed ''not AK'' denoted biopsies that did not meet the criteria for early AK or AK.

Immunohistochemistry. After deparaffinization, immunohistochemical staining was done using a streptavidin-biotin peroxidase system with a 3,3′-diaminobenzidine chromagen and a hematoxylin counterstain (Ventana Medical Systems, Tucson, AZ) on an automated VMS 320 immunostainer (Ventana Medical Systems). Anti-PCNA PC10, p53 (Oncogene Science, Uniondale, NY) was used at a 1:200 dilution in antibody diluent (Ventana Medical Systems). Negative controls (to assure lack of nonspecific staining) were run on each sample by substituting antibody diluent for the antibody. A tonsil tissue (PCNA) or the T45 breast cell line (p53) was included in each run as a positive control to assure proper staining and to assess variability of staining intensity. Tissue sections were measured on an ImagePro Plus (Media Cybernetics, Silver Springs, MD) and a Leica DMR microscope (Leica, Westzlar, Germany), and a Sony 3CCD color video camera (Sony, Tokyo, Japan). The percentage positive nuclear area per 20× field was determined for each biopsy.

Polyamine Analysis. All skin samples were placed immediately on ice and transported within 30 minutes to the laboratory where they were cut into ~1-mm pieces weighing ~5 mg each. After weighing, the samples were stored at −80°C until processed for polyamine assays. Skin biopsies were homogenized with 0.2 mol/L perchloric acid containing 10 μmol/L 1,7-diaminoheptane as an internal standard. The homogenate was centrifuged, and 100 μL of the supernatant were transferred to a new 1.5 mL Eppendorf tube containing 100 μL of 1 mol/L sodium carbonate. To the sample tube, 100 μL of 1% dansyl chloride in acetone were added and the sample was placed at 60°C for 1 hour. Fifty microliters of 10% glycine were added to remove excess dansyl chloride. After incubation for 30 minutes, dansyl polyamines were extracted with hexane. The extract was dried under nitrogen and redissolved with 125 μL acetonitrile. A 50 μL aliquot of the resulting solution was injected onto the high-performance liquid chromatography column. An Ultrasphere ornithine decarboxylase (ODC) 5 μm reversed-phase column (Beckman Instruments, Inc., San Ramon, CA; 4.6 × 250 mm) was used for analysis with a gradient of acetonitrile-disodium phosphate [1.2 mmol/L (pH 5.49)], a flow rate of 2.5 mL/min at room temperate, and a 7-minute run time. A Kratos Spectroflow 980 fluorescence detector (ABI Analytical, Inc., Remsey, NJ) provided detection with excitation at 340 nm and emission at 550 nm. The detection limit was <1 pmol, with linearity of up to 250 pmol for each polyamine injected. Recoveries for putrescine, spermidine, and spermine were 105%, 99%, and 81%, respectively. Additionally, all analytes were stable in skin stored at −80°C for at least 2 months.

Statistical Analysis. All analyses were conducted using Stata version 9 (StataCorp, College Station, TX). The values for p53 and PCNA expression were derived by averaging the three highest staining ×20 fields for each biopsy. Comparisons between baseline and 3-month samples only included those participants with biopsies from both time points. Because the initial analysis showed no significant differences between baseline and 3-month measurements, with the exception of PCNA in the sunscreen group, mean biomarker levels were calculated by averaging baseline and 3-month values for the comparisons between groups. Participants were included in these time-independent analyses as long as they had a single measurement at either time point. For comparison of average p53 or PCNA by histologic diagnosis, expression levels of all biopsies for a given histologic diagnosis were averaged to yield a single mean percentage of expression per subject. For normally distributed variables, paired *t* tests and ANOVA were used. For nonnormally distributed variables, Wilcoxon signed rank and Kruskal-Wallis tests were used.

Results

Participant Characteristics. Subjects were selected for inclusion in the three groups based on the dermatologist assessment of the presence of clinical sun damage, AK, or, in the case of the SCC group, a previously resected pathology verified SCC and age >18 years. An overview of the study design is shown in Fig. 1. Because groups were not randomized by age or gender, there were significant differences across the three groups. The mean ages (years ± SD) were 58.1± 10.4, 61.0 ± 9.9, and 65.9 ± 8.9 for group 1 (sun damage only), group 2 (presence of AK on forearms), and group 3 (prior history of SCC), respectively. The percentages of males in each group were 33.3%, 75.8%, and 62.6% in group 1, group 2, and group 3, respectively. Both PCNA and p53 were significantly higher in males than females (11.2 ± 0.9% versus 9.0 ± 0.8%, $P = 0.049$ for PCNA and 18.5 ± 1.6% versus 10.6 ± 1.3%, $P = 0.0001$ for p53). There were no significant differences in levels of polyamines by gender (data not shown). Subject age did not have a significant effect on any of the biomarkers and there were no significant differences between the groups for self-reported skin type (data not shown).

In the group with previously resected SCC, the SCCs were located on arms ($n = 20$), hand ($n = 1$), chest ($n = 4$), thigh ($n = 5$), and back ($n = 5$). A shave biopsy was done immediately adjacent to each site of the previous resection for PCNA and p53 expression.

In the second sample set (normal-appearing facelift and upper inner arm skin and AK) analyzed only for polyamines, the mean age in the AK group was 68.8 ± 7.5 years, in the group with normal-appearing skin from the facelift procedures was 61.5 ± 4.2 years, and in the group with upper inner arm skin was 68.4 ± 9.4 years. Gender in the AK group was 86.5% male, in the normal-appearing skin from the facelift procedures was 0% male, and in the upper inner arm skin was 85.7% male.

Differential Biomarker Expression. Biomarkers were first analyzed based on the dermatologist's clinical assessment (i.e., presence of clinical sun damage alone or AK). As seen in Fig. 2, in the forearm samples from each group, expression of p53 (mean ± SE) was significantly increased in the subjects with clinical evidence of AK (20.9 ± 2.3%) compared with the subjects with sun-damaged skin, but no evidence of clinical AK (11.5 ± 1.2%, $P = 0.0001$). The AK lesion itself (25.4 ± 2.6%) was not significantly different from its matching forearm samples. There was a significant difference between the sites adjacent to a previous SCC (10.0 ± 1.7%) and the matching forearms from those subjects (15.5 ± 2.5, $P = 0.02$). Furthermore, there was a significant difference between forearms from those with AK and those with a previous SCC ($P = 0.05$), but not between the forearms from those with a previous SCC and the forearms from those with sun damage alone ($P = 0.11$).

As also seen in Fig. 2, there was a nonsignificant increase in PCNA expression in the forearms of subjects with AK compared with the forearms of subjects with sun-damaged skin but no evidence of clinical AK (12.1 ± 1.6% versus 9.9 ± 0.7%, $P = 0.29$). PCNA expression in the AK lesion itself (16.1 ± 2.2%) did not reach statistical significance when compared with its matching forearm samples (12.1 ± 1.04, $P = 0.07$). There was a significant difference between the sites adjacent to a previous SCC (6.3 ± 0.8%) and the matching forearms from subjects with a previous SCC (8.5 ± 2.5%, $P = 0.04$).

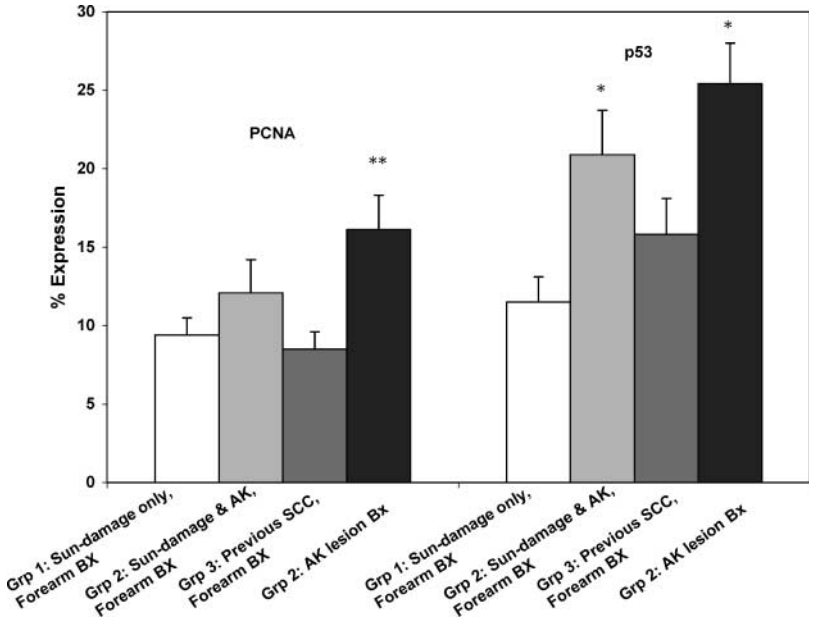

Figure 2. PCNA and p53 expression in forearm biopsies and AK lesion from subjects with sun-damaged skin, AK, and a previously resected SCC. ❑, group (*Grp*) 1 sun-damaged forearms ($n = 77$); ▒, group 2 sun-damaged forearms from subjects with AK ($n = 31$ for PCNA and 33 for p53); ■, group 3 forearms from subjects with previous SCCs ($n = 30$ for PCNA and 32 for p53); ■, AK lesion ($n = 31$ for PCNA and 33 for p53). *, $P \leq 0.0001$, difference between p53 in sun-damaged forearms versus forearms from subjects with AK and between sun-damaged forearms and the AK lesion. **, $P = 0.01$, the difference between PCNA in sun-damaged forearms and the AK lesion.

Figure 3 shows PCNA and p53 expression based on the histologic diagnosis of the biopsy rather than the clinical diagnosis. For p53, there was a stepwise and significant increase in the percentage of p53-positive cells from biopsies without characteristics of AK (i.e., normal appearing, 12.7 ± 1.1%) compared with early AK (16.4 ± 1.7%, $P = 0.02$), and early AK compared with the AK lesion (28.7 ± 3.0%, $P = 0.006$). Five SCCs were identified from the group of clinical AK and had a similar level of p53 expression (26.0 ± 13.4%) to that found in AK ($P = 0.34$). PCNA showed a similar pattern of expression with 9.1 ± 0.6%, 11.8 ± 1.2%, 16.9 ± 2.2%, and 10.4 ± 5.1% in biopsies without characteristics of AK (i.e., normal-appearing), early AK, AK, and SCC, respectively. The only significant difference was between early AK and AK ($P = 0.05$).

Figure 4 shows polyamine levels in the forearm biopsies from individuals with sun damage, AK, and a previously resected SCC. There was a significant increase in putrescine levels from 65.5 ± 1.9 nmol/g in sun-damaged skin to 81.7 ± 3.9 nmol/g ($P = 0.0002$) in the forearms of subjects with AK and in the forearms of subjects with a previously resected SCC (82.0 ± 3.5 nmol/g, versus sun-damaged, $P = 0.0001$). For putrescine, the forearm biopsies from subjects with AK and the forearms from those with a previous SCC were similar ($P = 0.4$). Spermidine levels were also significantly increased from 187.7 ± 3.3 nmol/g in sun-damaged skin to 209.4 ± 8.2 nmol/g in the forearms of subjects with AK ($P = 0.006$) and 204.8 ± 5.3 in the forearms of subjects with a previous SCC ($P = 0.005$). As seen with putrescine, spermidine concentrations were similar in the forearm biopsies from subjects with AK and forearms of those with a previous SCC ($P = 1.0$). With respect to spermine concentrations, the sun-damaged forearm biopsies were significantly different from the forearm biopsies from subjects with AK ($P = 0.01$) and from the AK lesions ($P = 0.001$).

Figure 4 also contains the results from a separate group of samples that were obtained from AK lesions in 37 subjects with clinical AK on forearms and normal skin from 20 individuals undergoing plastic surgery and from 14 subjects where the upper inner arm was sampled. There were no significant differences in the polyamine levels between the normal skin obtained from plastic surgery and from the upper inner arm with the exception of a significant difference in spermidine concentrations in facelift skin (162.0 ± 8.6 nmol/g) compared with upper inner arm skin (129.0 ± 13.4 nmol/g, $P = 0.005$). Putrescine levels were 120.8 ± 7.7

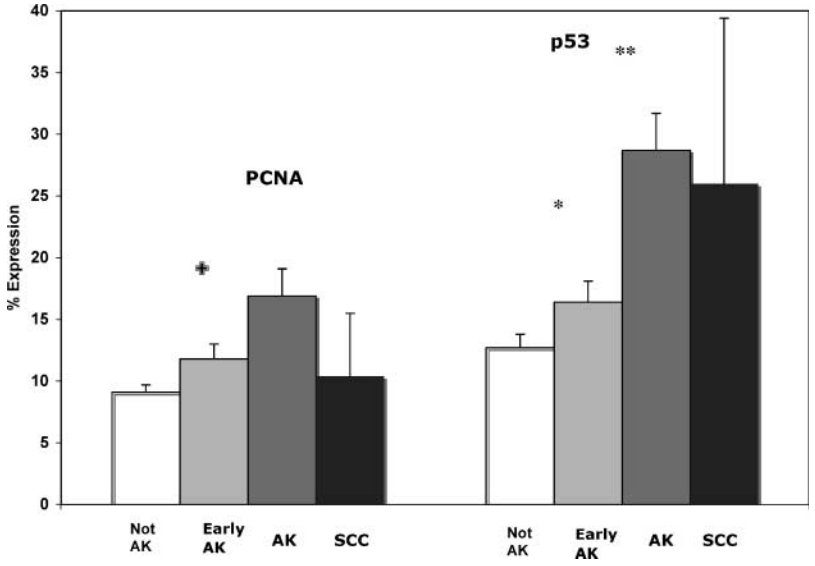

Figure 3. PCNA and p53 expression in biopsies histologically confirmed as not meeting the criteria of an AK, early AK, and AK. ❑, not AK ($n = 134$ for PCNA and 135 for p53); ▒, early AK ($n = 68$ for PCNA and 69 for p53); ▒, AK ($n = 34$ for PCNA and 37 for p53); ■, SCC ($n = 4$ for PCNA and 5 for p53). *, $P = 0.02$ for p53 expression, not AK versus early AK. **, $P = 0.0001$ for p53 expression, early AK versus AK; ✙, early AK versus AK. **, $P = 0.05$ for PCNA expression.

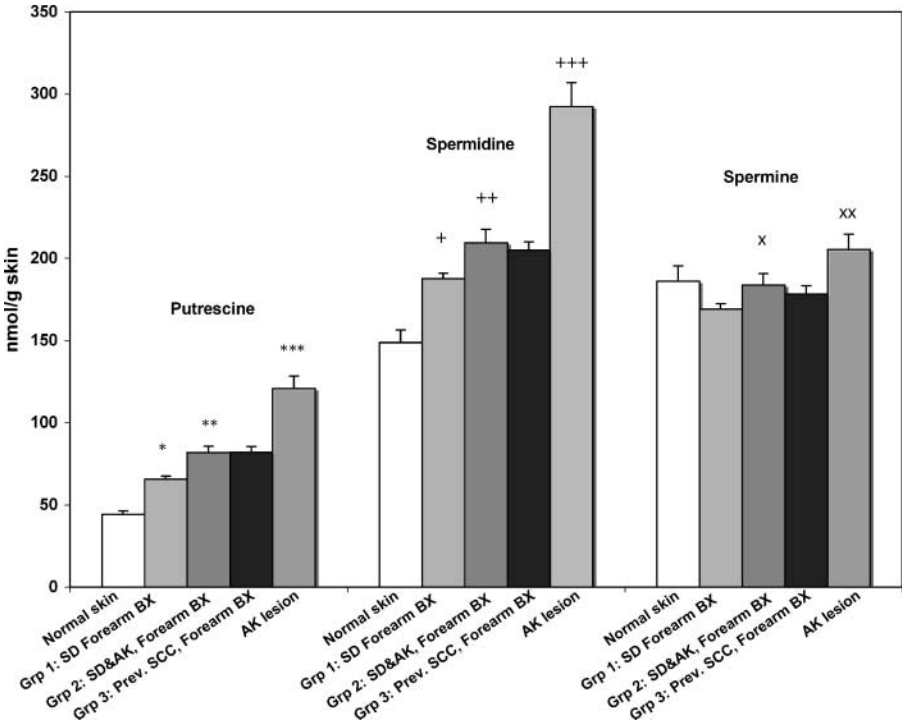

Figure 4. Polyamine levels in normal skin, sun-damaged forearm, forearm of subjects with AK, forearm of subjects with prior SCC, and AK. ❑, normal upper inner arm or face-lift skin (n = 34); ■, group 1 sun-damaged (*SD*) forearms (n = 42); ■, group 2 SD forearms from subjects with AK (n = 22); ■, group 3 forearms from subjects with previous SCCs (n = 28); ■, AK lesions (n = 37). For putrescine: *, $P < 0.0001$, normal skin versus sun-damaged forearm; **, $P < 0.002$, sun-damaged forearm versus AK forearm; ***, $P < 0.0001$, AK forearm versus AK lesion. For spermidine: +, $P < 0.0001$, normal skin versus sun-damaged forearm; ++, $P < 0.006$, sun-damaged forearm versus AK forearm; +++, $P < 0.0001$, AK forearm versus AK lesion. For spermine: ×, $P < 0.01$, sun-damaged forearm versus AK forearm; ××, $P = 0.001$, sun-damaged forearm versus AK lesion.

nmol/g in the AK lesion and 44.1 ± 2.3 nmol/g in normal skin (P = <0.0001), whereas spermidine levels were 292.2 ± 14.8 nmol/g in AK and 148.7 ± 7.9 nmol/g in normal skin ($P < 0.0001$). Spermine levels were 205.5 ± 9.8 nmol/g in AK and 186.1 ± 9.1 in normal skin (P = 0.15). When we compared polyamine concentrations in the groups of forearm samples to this separate sample set of normal and AK lesions, the forearms of subjects with sun damage had significantly higher levels than the normal skin ($P < 0.0001$), and the AK lesions had significantly higher levels than the forearm biopsies from subjects with an AK for both putrescine and spermidine ($P < 0.0001$). The only significant difference found for spermine was between the AK lesion and the forearms of subjects with sun damage (P = 0.01).

Biomarker Reliability. Table 1 shows the results of p53 expression at baseline and at the 3-month time point in the three groups. There were no significant differences in any of the groups. In addition, in the sun-damaged group 1, the use of sunscreen did not significantly affect p53 expression. Similar results were seen for PCNA expression (Table 2) with the exception of the sun-damaged group randomized to no sunscreen (P = 0.005) where there was a significant decrease in PCNA expression.

Table 3 shows the results for individual polyamine levels at baseline and at the 3-month time point. In contrast to p53 and PCNA expression, only forearm biopsies were obtained at the two time points for polyamines. There were no significant differences in any of the groups in the baseline compared with the 3-month time point, and in addition the use of sunscreen did not significantly affect polyamine levels.

Discussion

UV-induced human SCC is a multistep process where normal sun-exposed skin progresses to sun-damaged skin followed by the premalignant AK and malignant SCC stages. Although it is unlikely that all SCCs will develop via this pathway, the vast majority will (19). Even though AK are often used as surrogates for SCC in chemoprevention trials, there remains a scarcity of validated biomarkers in this area (17). In this study, we addressed several important issues regarding the use of biomarkers in chemoprevention trials, including expression in the different stages of UV-induced skin tumorigenesis, repeat biopsies, and use of sunscreen.

We evaluated p53 and PCNA expression and polyamine content in groups of subjects and samples that represented the

Table 1. p53 expression at baseline and at 3 months in forearm biopsies, AK lesions, and skin adjacent to previous SCC

Group	Sample	n	Mean % p53 expression (SE)	P*
Group 1: sun-damaged forearm, no sunscreen	Baseline	33	12.95 (2.80)	0.30
	3 mo	33	10.37 (2.53)	
Group 1: sun-damaged forearm, sunscreen	Baseline	43	12.28 (1.95)	0.79
	3 mo	43	11.07 (1.56)	
Group 2: AK forearm	Baseline	32	19.54 (2.88)	0.59
	3 mo	32	21.48 (3.03)	
Group 2: AK lesion	Baseline	30	27.18 (3.94)	0.47
	3 mo	30	21.81 (3.62)	
Group 3: skin adjacent to previous SCC	Baseline	30	10.51 (2.30)	0.12
	3 mo	30	8.5 (2.25)	
Group 3: forearm previous SCC forearm	Baseline	32	15.41 (2.66)	0.99
	3 mo	32	15.56 (2.92)	

*Wilcoxon signed rank test comparing baseline expression to 3 months expression.

Table 2. Mean percentage of PCNA expression at baseline and at 3 months in forearm biopsies, AK lesions, and skin adjacent to previous SCC

Group	Sample	*n*	Mean % PCNA (SE)	*P**
Group 1: sun-damaged forearm, no sunscreen	Baseline	33	12.8 (1.8)	0.0049
	3 mo	33	7.8 (1.1)	
Group 1: sun-damaged forearm, sunscreen	Baseline	43	11.1 (1.4)	0.19
	3 mo	43	8.3 (0.9)	
Group 2: AK forearm	Baseline	30	12.0 (2.2)	0.39
	3 mo	30	12.9 (2.6)	
Group 2: AK lesion	Baseline	27	14.7 (2.5)	0.32
	3 mo	27	18.9 (3.7)	
Group 3: skin adjacent to previous SCC	Baseline	30	6.5 (1.0)	0.19
	3 mo	30	4.7 (1.0)	
Group 3: previous SCC forearm	Baseline	32	7.3 (1.1)	0.13
	3 mo	32	9.6 (1.4)	

*Wilcoxon signed rank test comparing baseline expression with 3 months expression.

continuum of early UV-induced tumorigenesis (i.e., subjects with sun damage but no clinical evidence of AK on forearms, subjects with AK on forearms, and subjects with a previously resected SCC). When data were analyzed by the site and clinical impression of the skin where the biopsy was obtained, we found significant differences in p53 expression and polyamine concentrations (putrescine and spermidine) in the forearm skin of the group with sun damage alone compared with the group with clinical evidence of AK on forearms. Both p53 expression and polyamine concentrations were further increased in the AK lesion itself compared with sun-damaged skin alone. Moreover, when the histology of the lesion was taken into consideration, there was a stepwise and significant increase in p53 expression from biopsies without characteristics of AK (i.e., normal appearing) compared with early AK and AK.

To our knowledge, this is the first report of significant differences in the expression of p53 in the forearms of groups of individuals with sun damage alone versus those with the presence of AK, presumably more progressed along the UVB-induced skin cancer pathway. In contrast, there were no significant differences in p53 expression in the matching forearm skin from subjects with AK compared with the AK lesion itself. Carpenter et al. (20) used a similar study design where they sampled an AK and matching nearby sun-damaged skin and found significant differences in p53 expression and a marker of proliferation, Ki-67. The reason for the discrepancy in these two studies is unclear, but may be due to slight differences in the populations at the two study locations, small differences in study design, as well as technical differences in biomarker measurement. In previous studies, we have shown that p53 was significantly different in skin directly adjacent to an AK compared with the matching AK. Our previous studies were also from a somewhat different study population that was primarily recruited from the

Table 3. Polyamine levels at baseline and 3 months in forearm biopsies from subjects with sun damage, AK, or a previous SCC

Group	Sample	*n*	Average (SE)	*P**
Group 1: sun-damaged forearm, no sunscreen	Putrescine baseline	20	62.65 (4.32)	0.3221
	Putrescine 3 mo	20	68.10 (2.97)	
	Spermine baseline	20	157.36 (5.72)	0.2100
	Spermine 3 mo	20	168.46 (6.40)	
	Spermidine baseline	20	178.00 (6.36)	0.3776
	Spermidine 3 mo	20	188.02 (7.83)	
	Total baseline	20	398.00 (14.44)	0.3051
	Total 3 mo	20	440.06 (30.41)	
Group 1: sun-damaged forearm, sunscreen	Putrescine baseline	22	65.31 (4.34)	0.8785
	Putrescine 3 mo	22	66.07 (3.06)	
	Spermine baseline	22	172.67 (8.07)	0.6717
	Spermine 3 mo	22	176.91 (4.49)	
	Spermidine baseline	22	186.26 (8.75)	0.2904
	Spermidine 3 mo	22	197.53 (4.67)	
	Total baseline	22	424.25 (18.96)	0.5645
	Total 3 mo	22	444.91 (21.30)	
Group 2: AK forearm	Putrescine baseline	22	82.21 (4.92)	0.7771
	Putrescine 3 mo	22	81.17 (3.71)	
	Spermine baseline	22	182.27 (6.95)	0.5674
	Spermine 3 mo	22	185.16 (7.81)	
	Spermidine baseline	22	209.59 (8.70)	0.9558
	Spermidine 3 mo	22	209.26 (8.78)	
	Total baseline	22	474.07 (19.41)	0.9939
	Total 3 mo	22	474.23 (23.32)	
Group 3: previous SCC forearm	Putrescine baseline	28	81.66 (4.27)	0.9017
	Putrescine 3 mo	28	82.30 (4.43)	
	Spermine baseline	28	177.71 (5.81)	0.9060
	Spermine 3 mo	28	178.65 (7.06)	
	Spermidine baseline	28	204.57 (5.66)	0.9593
	Spermidine 3 mo	28	204.96 (7.30)	
	Total baseline	28	463.94 (13.40)	0.9218
	Total 3 mo	28	466.95 (26.58)	

*Paired *t* tests.

Southern Arizona Veterans Medial Center, used a smaller sample size ($n = 19$) and a different study design (skin directly adjacent to an AK compared with its matching AK; refs. 21, 22).

The *p53* gene is clearly essential in the maintenance of genomic integrity through a blockage of DNA replication in response to DNA damage due to exposure to agents like UV light. Cells with extensive damage are blocked from entering the cell cycle and instead undergo apoptosis (15, 16). Mutation of p53 disrupts this process, often leading to a stabilization of the protein (12, 13, 16, 23-31). We previously reported that p53 expression was in fairly good agreement with the presence of p53 mutations (21) and that the frequency of p53 mutations and expression increases between upper inner arm skin, skin adjacent to an AK, but that AK and SCC were similar (22). Moreover, we have previously reported that topical difluoromethylornithine treatment significantly reduced p53 expression in skin to a similar degree as the reduction in AK number (32). A recent abstract by Van der Pols et al. (33) described the results of a cross-sectional study showing that p53 expression in baseline biopsies of the hand predicted the subsequent risk of SCC, but not basal cell carcinoma, at a 7-year follow-up.

Although polyamines and ODC activity have been shown to be increased after UV irradiation in human skin, we believe that this is the first report demonstrating that polyamine concentrations are elevated in a significant and stepwise fashion in normal nonexposed skin, sun-damaged skin, and clinical AK (34-36). Polyamines are multifunctional and involved in cell proliferation, cell survival, cell signaling, and apoptosis (37). Khettab et al. (38) showed a significant increase in the production of free radicals and polyamine biosynthesis in the epidermis of mice after UV, suggesting one potential mechanism for this up-regulation of polyamines in sun-exposed skin, although elevated polyamine concentrations and ODC activity have been implicated in skin tumorigenesis (39, 40). O'Brien et al. (39) showed that in transgenic mice, overexpression of ODC in skin was associated with elevated tissue polyamines, particularly putrescine and the development and maintenance of the neoplastic phenotype (40). ODC has also been found to be elevated in SCC compared with normal skin (41-43).

Although we did not observe significant differences between sun-damaged forearms and forearms from subjects with AK, PCNA expression was significantly different in AK compared with sun-damaged skin, a finding that we have published previously (44). Unlike p53 expression and polyamine levels, we have not been able to detect a modulation of PCNA expression by a chemopreventive agent like difluoromethylornithine (32). Further study will be needed to determine whether PCNA expression, or another measure of proliferation, will prove to be a reliable and useful biomarker in skin cancer chemoprevention trials. An index of the number of proliferative cells in the context of the number of apoptotic cells may well be more useful as a biomarker in these kinds of studies.

We also evaluated the reproducibility of p53 and PCNA expression and polyamine content in baseline biopsies compared with a 3-month time point. This time frame was chosen given that our phase I and phase II skin chemoprevention trials generally vary in length from 1 to 6 months. Another critical concern is whether the use of sunscreen confounds the results of skin cancer chemoprevention trials and in particular the expression of biomarkers. Notably, no significant differences were seen in the biomarkers in the baseline compared with the 3-month biopsies, with the exception of PCNA, which was reduced at the 3-month time point in the sun damage group only without sunscreen. This drop in PCNA was most pronounced in males reporting high levels of sun exposure. We found no obvious explanation for this finding and attribute it to a type 1 error due to the small sample size and inherent variability of the PCNA measurement.

Use of sunscreens in skin cancer chemoprevention trials is clearly an important issue that can confound trial results. Animal studies have shown that sunscreens can inhibit UV-induced skin carcinogenesis (45-47). In addition, Ananthaswamy et al. (47), showed that sunscreens inhibit UV-induced *p53* mutations as well as skin cancer development in mice. When incorporating biomarkers like p53 expression into skin cancer chemoprevention trials, potential confounding factors must be considered in the design of the trial. These may include recent sun exposure, use of sunscreen, or other sun protection measures along with the potential effects of the chemopreventive agent.

In conclusion, the current study adds validity for use of p53 expression and individual polyamine concentrations as biomarkers in skin cancer chemoprevention trials using a population with AK or sun-damaged skin. There also appears to be potential for use of biomarkers like p53 expression as predictive markers of future skin cancer risk, although this is an area that deserves further exploration. Study design is another important consideration. Although some skin cancer chemoprevention trials have used subjects as their own control (48, 49), others have relied on separate control groups (50). Both designs have utility with the goal to show clinical improvement, as well as a change in the biomarker as a result of an intervention.

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Janine G. Einspahr, Min-Jian Xu, James Warneke, et al.

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