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## Caffeine decreases phospho-Chk1 (Ser<sup>317</sup>) and increases mitotic cells with cyclin B<sub>1</sub> and caspase 3 in tumors from UVB treated mice

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### Abstract

Oral administration of caffeine to mice inhibits UVB-induced carcinogenesis, and these results are paralleled by epidemiology studies indicating that caffeinated coffee and tea intake (but not decaffeinated beverage intake) is associated with decreased incidence of nonmelanoma skin cancer. Topical applications of caffeine to the skin of SKH-1 mice that had previously been treated with UVB inhibited subsequent skin tumor development and stimulated apoptosis in tumors but not in non-tumor areas of the epidermis. The present study sought to determine the basis of these differential effects on tumor vs non-tumor sites that can be induced by caffeine long after all UVB treatment has ceased. The activation status of the ATR/Chk1 pathway in UVB-induced tumors and uninvolved skin was determined by quantitating phospho-Chk1 (Ser317) and induction of lethal mitosis *in vivo* in the presence and absence of topical caffeine treatment. In the absence of caffeine, we found that UVB-induced tumors often had islands of phospho-Chk1 (Ser317) staining cells that were not present in non-tumor areas of the epidermis. Treatment of mice with topical caffeine significantly diminished phospho-Chk1 (Ser317) staining and increased the number of mitotic cells that expressed cyclin B1 and caspase 3 in tumors, consistent with caffeine-induced lethal mitosis selectively in tumors. We hypothesize that compared to adjacent uninvolved skin, UVB-induced skin tumors have elevated activation of, and dependence on, the ATR/Chk1 pathway long after UVB exposure has ceased and that caffeine can induce apoptosis selectively in tumors by inhibiting this pathway and promoting lethal mitosis.

### Keywords

sunlight-induced skin cancer; cancer chemoprevention; enhanced lethal mitosis

### Introduction

There are more than one million cases of nonmelanoma skin cancers per year in the United States [1], and the number of these cancers has been increasing in recent years [2, 3]. Accordingly, a better understanding of approaches for the prevention of these cancers is

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important. Several studies have demonstrated an inhibitory effect of caffeine administration on UVB-induced skin carcinogenesis in mice [4-7], and these studies are paralleled by epidemiology studies that indicate an inhibitory effect of coffee ingestion on sunlight-induced nonmelanoma skin cancer in humans while decaffeinated coffee had no such effect [8, 9]. Analogous studies in mice indicate a stimulatory effect of oral caffeine or coffee administration on UVB-induced apoptosis in SKH-1 mice [10, 11].

In recent mechanistic studies we found that a single irradiation of mouse skin with UVB activated the ATR/Chk1 pathway causing a several-fold increase in epidermal phospho-Chk1 (Ser345) and a marked decrease in mitotic epidermal cells with cyclin B1 compared to baseline [12]. Administration of caffeine in the drinking water for 1 or 2 weeks before UVB irradiation markedly inhibited the UVB-induced increase in phospho-Chk1 (Ser345) and caused a premature increase in cyclin B1 that was associated with increased apoptosis [12]. A single irradiation with UVB in p53 knockout mice also markedly decreased the number of mitotic epidermal cells with cyclin B1, and topical application of caffeine immediately after UVB abrogated this response and increased UVB-induced apoptosis several-fold [13]. These effects of caffeine in p53 knockout mice were substantially greater than in their p53 wild-type littermates, suggesting that modest doses of caffeine *in vivo* can selectively sensitize p53-deficient cells to apoptosis after UVB. In an additional study with cultured human keratinocytes, decreasing ATR or Chk1 with siRNAs enhanced UVB-induced apoptosis, and this effect was not further increased by the addition of caffeine, suggesting that caffeine was acting via ATR/Chk1 pathway suppression [14]. These observations provide further support for the view that caffeine exerts its effect to enhance UVB-induced apoptosis by inhibiting the ATR/Chk1 pathway.

In an additional study we treated SKH-1 mice with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks. These mice had no tumors, but they had a high risk of developing tumors during the next several months in the absence of further irradiation with UVB ("high-risk mice") [6]. Topical applications of caffeine five days a week for 18 weeks to these "high-risk mice" diminished the development of malignant tumors by 72% and selectively increased apoptosis in the tumors but not in areas of the epidermis away from the tumors [7]. Mechanisms of this effect of caffeine on UVB-induced tumors were not previously evaluated and are the topic of the present manuscript.

Recent studies demonstrated that dysregulation of DNA replication by overexpression of certain oncogenes and growth factor receptors induces DNA replication stress and stalled replication forks that result in activation of the ATR/Chk1 signaling pathway (formation of phospho-Chk1 at Ser 317 and Ser 345 and phospho-Rad17 at Ser 645) [15, 16]. These studies further suggest that collapsed replication forks tend to occur mainly at chromosomal fragile sites, induce DNA double-strand breaks, and activate the ATM/Chk2 signal transduction pathway. Precancerous lesions and cancers of the bladder, breast, colon and lung showed constitutive activation of ATM/Chk2 and stain positively for markers of DNA damage such as gamma-H2AX, phospho-ATM and phospho-Chk2 [15, 16]. In addition, an aberrant replication stress response resulting in constitutive activation of the ATR/Chk1 pathway was recently observed in human gliomas (increased phospho-Chk1 (Ser317)) [17]. The results of these studies [15-17] suggest a dysregulation of DNA replication during tumorigenesis and in tumors resulting in activation of ATR and ATM signal transduction pathways leading to cell cycle arrest. In the absence of a functional p53 pathway (as in UVB-induced tumors or UVB-induced precancerous lesions with p53 mutations), we anticipate activation of the ATR→Chk1→cdc25C→cdc2→cdc2/cyclin B1 pathway that should normally result in a decrease in cdc2/cyclin B1 and an arrest prior to mitosis. In the presence of caffeine, this arrest should be abrogated (cdc2/cyclin B1 activity should be increased despite DNA damage) and cells should proceed into mitosis prematurely leading

to p53-independent cell death – probably by mitotic catastrophe followed by apoptosis in early cancer cells and in epidermal tumors. We hypothesize that UVB-induced skin tumors will have an elevated expression of the ATR/Chk1 pathway that can be inhibited by caffeine and that the proapoptotic effect of caffeine in these tumors is due to inhibition of the DNA damage-dependent activation of the ATR/Chk1 pathway. In accord with this hypothesis, the results of the present study indicate that caffeine administration decreases phospho-Chk1 (Ser317) in UVB-induced tumors and increases the percentage of morphologically distinct mitotic tumor cells with cyclin B1 and caspase 3.

## Materials and Methods

### Chemicals and Animals

Acetone (HPLC grade) and 10% phosphate-buffered formalin were obtained from Fisher Scientific, caffeine (>99% pure) was obtained from Sigma. Female SKH-1 hairless mice (6–7 weeks old) were purchased from the Charles River Breeding Laboratories, and the animals were kept in our animal facility for at least 1 week before use. Mice were given water and Purina Laboratory Chow 5001 diet from Ralston-Purina (St. Louis) ad libitum, and they were kept on a 12-h light/12-h dark cycle.

### Treatment of Mice with UVB

The UV lamps used (FS72T12-UVB-HO; National Biological, Twinsburg, OH) emitted UVB (280–320 nm; 75–80% of total energy) and UVA (320–375 nm; 20–25% of total energy). There was little or no radiation below 280 nm or above 375 nm. The dose of UVB was quantified with a UVB Spectra 305 dosimeter (Daevlin, Byran, OH). The radiation was further calibrated with a model IL-1700 research radiometer/photometer (International Light, Newburyport, MA).

Mice were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks, and UVB treatment was stopped. Three weeks later, these tumor-free high-risk mice were randomized and divided into two groups (30 mice per group). Each group was treated topically on the back with 100 µl acetone or caffeine (1.2 mg, 6.2 µmol) in 100 µl acetone once a day 5 days a week for 18 weeks [7]. The mice were killed at 24 h after the last application of caffeine. Tumors on the treated areas of the mice were counted and characterized by histological examination.

### Preparation of Skin Sections

For histopathology examination and immunohistochemical analysis, the animals were killed and the dorsal skins were taken to include each of the grossly observed masses in the treated areas of the mice. The skins were stapled flat to a plastic sheet and placed in 10% phosphate-buffered formalin at 4°C for 24 h. The skin samples were then dehydrated in ascending concentrations of ethanol (80%, 95%, and 100%), cleared in xylene, and embedded in Paraplast (Oxford Labware, St. Louis). Four-micrometer serial sections of skin were made, deparaffinized, rehydrated with water, and used for regular hematoxylin-eosin staining or immunohistochemical staining. The counting and characterization of all tumors was done blinded with respect to treatment group as described [6, 7]. Unless otherwise specified, all immunohistochemical determinations were made with 400-fold magnification with a light microscope using stored paraffin blocks from a previous study [7]. These examinations were also done blinded with respect to treatment group.

### Phospho-Chk1 (Ser317) immunostaining

Skin sections used for the measurement of phospho-Chk1 (Ser317) were stained by the horseradish peroxidase-conjugated-avidin method with some modification. Endogenous

peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections were then treated with 0.01M sodium citrate buffer (pH 6.0) in a microwave oven at a high setting for 10 min. The sections were incubated with a protein block (normal goat serum) for 10 min, followed by avidin D for 15 min and biotin blocking solution for 15 min (Avidin-Biotin blocking kit from Vector Laboratory, Burlingame, CA) at room temperature. The sections were incubated with phospho-Chk1 (Ser317) primary antibody (cat. No:2344 purchased from Cell Signaling Technology Inc. Beverly, MA) for 30 min (1:50 dilution) at room temperature followed by incubation with a biotinylated anti-rabbit secondary antibody for 30 min and incubation with conjugated-avidin solution (ABC elite kit purchased from Vector Laboratory) for 30 min. Color development was achieved by incubation with 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The sections were then counterstained with hematoxylin, dehydrated, and coverslips were added for permanent mounting. A positive reaction is shown as a light brown to dark brown precipitate in the cytoplasmic and/or perinuclear portion of the cells. The percentage of phospho-Chk1 (Ser317) positive cells in the epidermis was calculated from the number of phospho-Chk1 (Ser317) stained cells divided by the total number of tumor cells counted from the entire areas of tumor section.

### Mitotic cells with caspase 3-positive staining

Affinity-purified polyclonal rabbit antibody that reacts with the mouse p20 subunit of caspase 3 but does not react with the precursor form was purchased from R&D Systems. Skin sections used for the measurement of caspase 3 were stained by the horseradish peroxidase-conjugated-avidin method with some modification. Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections were then treated with 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven at high temperature for 10 min. The sections were incubated with a protein block (normal goat serum) for 10 min, followed by avidin D for 15 min and biotin blocking solution for 15 min (Avidin-Biotin blocking kit from Vector Laboratories) at room temperature. The sections were incubated with caspase 3 primary antibody (1:2,000 dilution) for 30 min at room temperature followed by incubation with a biotinylated anti-rabbit secondary antibody for 30 min and incubation with conjugated-avidin solution (ABC elite kit purchased from Vector Laboratories) for 30 min. Color development was achieved by incubation with 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The slides were then counterstained with hematoxylin, dehydrated, and coverslips were added for permanent mounting. A positive reaction was shown as a light brown to dark brown precipitate in the cytoplasm and/or perinuclei of the cells. The percent of caspase 3-positive cells was determined in each lesion. In nontumor areas, the scoring was performed at least 0.5 cm away from tumors.

Mitotic cells were determined as described earlier [12, 13] by observing (a) chromosome condensation together with breakdown of the nuclear envelope, (b) alignment of the chromosomes on the spindle equator, (c) separation of sister chromatids and (d) movement to their respective spindle poles. The entire tumor sections were examined, and the percentage of total tumor cells that were mitotic with caspase 3 positive staining was analyzed.

### Mitotic cells with cyclin B1-positive staining

Cyclin B1 immunostaining was described earlier [12]. Briefly, skin sections were first treated with 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven at high setting for 15 min. The sections were then incubated with a protein block followed by incubation with cyclin B1 antibody (purchased from Abcam Inc., Cambridge, MA, cat. No: ab72) at 1:500

dilution for 30 min at room temperature. The antibody for cyclin B1 is a mouse monoclonal antibody produced by immunizing the mouse with a His-tagged hamster cyclin B1 expressed in bacteria. This antibody is specific for cyclin B1 and is recommended by the manufacturer for the immunohistochemical detection of cyclin B1 in mouse tissues. The samples were then incubated with a biotinylated anti-rabbit secondary antibody for 5 min at 37°C, followed by incubation with conjugated streptavidin solution for 5 min at 37°C. Color development was achieved by incubation with 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The slides were then counterstained with hematoxylin and dehydrated. A positive reaction was shown as a brown precipitate in the cells (staining in nucleus and cytoplasm). The percentage of total tumor cells that were mitotic with cyclin B1 positive staining was analyzed. The percentage of mitotic cells that had positive staining for cyclin B1 was also calculated.

## Results and Discussion

Oral administration of caffeine to SKH-1 mice inhibits UVB-induced carcinogenesis [5, 6], and these results are paralleled by epidemiology studies indicating that coffee drinkers have a decreased risk of nonmelanoma skin cancer [8, 9]. In the present study, we evaluated the effect of topical applications of caffeine during the course of tumor formation in UVB-pretreated high risk mice on the ATR/Chk1 pathway in UVB-induced tumors.

### Topical applications of caffeine decrease the level of phospho-Chk1 (Ser317) in tumors from UVB-pretreated high-risk mice

Keratoacanthomas and squamous cell carcinomas that formed in UVB-pretreated high-risk mice had focal islands of phospho-Chk1 (Ser317)-staining cells indicating activation of the ATR/Chk1 pathway (Fig 1A), but these were not observed in 641 “normal-appearing” areas of the epidermis away from tumors (~215,000 cells) (Fig. 1A) or in 44 focal hyperplastic areas of the skin (data not presented). We hypothesize that administration of caffeine after stopping UVB will enhance apoptosis and inhibit carcinogenesis in UVB-pretreated high-risk mice by decreasing the level of phosphorylated Chk1 in tumors. In the present study of UVB-pretreated high-risk SKH-1 mice [7] described in Table 1, phospho-Chk1 (Ser317) was measured immunohistochemically in 59 large keratoacanthomas (selected from 18 mice from the acetone-treated control group) and in 50 size-matched keratoacanthomas from 13 mice in the caffeine-treated group, as well as in all of the squamous cell carcinomas from both groups. Focal islands of cells with phospho-Chk1 (Ser317) were observed in many but not all tumors (Table I). Representative focal islands of cells from control animals that have elevated phospho-Chk1 (Ser317) expression in a keratoacanthoma and a squamous cell carcinoma are shown in Fig. 1A. It was of interest that expression of phospho-Chk1 (Ser317) was almost exclusively in the cytoplasm (Fig. 1B). Topical treatment of these high-risk mice with caffeine for 18 weeks decreased significantly the number of phospho-Chk1 (Ser317) positive keratoacanthomas by 56% and the percentage of phospho-Chk1 (Ser317) positive cells in keratoacanthomas by 64% (Table 1). The intensity of staining for phospho-Chk1 (Ser317) in keratoacanthomas was also markedly decreased in caffeine-treated mice (Table 1, Fig. 1B). Although there was little or no decrease in the percentage of phospho-Chk1 (Ser317) positive cells in squamous cell carcinomas (Table 1), the intensity of staining was markedly decreased (Table 1, Fig. 1B). Our results indicate that topical applications of caffeine decreased the level of phospho-Chk1 (Ser317) in both keratoacanthomas and squamous cell carcinomas, and the effect of caffeine was greater in keratoacanthomas.

We used the Pearson's correlation coefficient for evaluating the relationship between the percentage of phospho-Chk1 (Ser317) positive cells and the percentage of caspase 3 positive cells in individual tumors from acetone-treated control mice and in individual tumors from



mice treated with caffeine. We found a weak but statistically significant inverse relationship between the percentage of phospho-Chk1 (Ser317) positive cells and the percentage of caspase 3 positive cells in tumors. In combined keratoacanthomas, from acetone-treated control mice and caffeine-treated mice, the  $r$  was  $-0.273$  ( $p < 0.01$ ). In combined squamous cell carcinomas from acetone-treated control mice and caffeine-treated mice, the  $r$  was  $-0.286$  ( $p < 0.05$ ). Our results indicate a statistically significant relationship between phospho-Chk1 (Ser317) and apoptosis in the tumors.

### **Topical applications of caffeine increase the percentage of mitotic cells with caspase 3 (active form) in tumors from UVB-pretreated high-risk mice**

In an earlier study, topical applications of caffeine to UVB-pretreated high-risk mice in the absence of further treatment with UVB inhibited skin tumorigenesis, and the mechanism of this inhibition by caffeine – at least in part – is by induction of apoptosis selectively in the tumors but not in non-tumor areas of the epidermis [7]. Topical applications of caffeine to UVB-pretreated high-risk mice decreased the number of nonmalignant and malignant skin tumors per mouse by 44% and 72%, respectively [7]. Treatment of the mice with caffeine increased apoptosis as measured by the number of caspase 3-positive cells in keratoacanthomas by 87% and in squamous cell carcinomas by 92% [7]. These effects on apoptosis were selective for tumors since treatment of the mice with caffeine had little or no effect on caspase-3 positive cells in non-tumor areas of the epidermis [7].

In the present study, we observed that some mitotic cells in the tumors were undergoing apoptosis as indicated by staining for caspase 3 (active form) (Fig. 2, left panel). Treatment of high-risk mice with caffeine topically for 18 weeks increased the percentage of keratoacanthoma cells with both mitosis and caspase 3 (active form) staining by 209% ( $p < 0.01$ ), and this treatment also increased the percentage of squamous cell carcinoma cells with both mitosis and caspase 3 (active form) staining by 212% ( $p < 0.01$ ), when compared with acetone-treated control animals (Table 2). Treatment with caffeine increased the percentage of morphologically distinct mitotic cells with caspase 3 staining from 7% in control keratoacanthomas to 22% in caffeine treated tumors (214% increase; Table 2; Fig. 3). Treatment with caffeine increased the percentage of morphologically distinct mitotic cells with caspase 3 staining from 6% in control squamous cell carcinomas to 25% in caffeine-treated tumors (317% increase; Table 2; Fig 3). In contrast to these results, very few morphologically distinct mitotic cells were observed in areas of the epidermis away from tumors, and treatment with caffeine had no significant effect on the percentage of mitotic cells with caspase 3 (Table 2, Fig. 3). Our results indicate for the first time that caffeine administration increased the number of tumor cells undergoing lethal mitosis, but this was not observed in areas of the epidermis away from tumors.

### **Topical applications of caffeine increase the percentage of mitotic cells with cyclin B1 in tumors from UVB-pretreated high-risk mice**

Since caffeine administration decreased the level of phospho-Chk1 (Ser317) and increased the number of mitotic cells with caspase 3 in UVB-induced tumors, we explored the effect of caffeine on the level of cyclin B1 in these tumors. Cyclin B1, which participates in the regulation of mitosis, complexes with p34(cdc2) to form the maturation-promoting factor (mitosis-promoting factor) that is expressed predominantly during the G2/M phase of the cell cycle. During the G1, S, and G2 phases, an antibody to cyclin B1 labels the cytoplasm. In prophase, labeled cyclin B1 translocates from the cytoplasm to the nucleus. The nuclear labeling then dissipates during the later stages of mitosis. Examples of mitotic cells that also stain for cyclin B<sub>1</sub> are shown in Fig. 2 (right panel). In the present study, we determined the effects of caffeine on the percentage of tumor cells that were mitotic with cyclin B1 positive staining. We evaluated the possibility that topical caffeine enhanced apoptosis through

abrogation of the cell cycle blockade at the G2/M checkpoint by elevating the promitotic cyclin B1 level prematurely thereby causing premature and lethal mitosis in the tumors.

We measured cyclin B1 in 38 large keratoacanthomas (selected from 15 mice from the acetone-treated control group) and in 28 size-matched keratoacanthomas (from 15 mice from the caffeine-treated group). We observed that topical applications of caffeine to UVB-pretreated high-risk mice caused a statistically significant 66% increase ( $p < 0.01$ ) in the percentage of morphologically distinct mitotic cells with cyclin B1 (Table 3). Treatment with caffeine increased the percentage of mitotic cells with cyclin B1 staining from 10% in control keratoacanthomas to 17% in caffeine treated tumors (70% increase; Table 3). Our results indicate that treatment with caffeine increased the level of cyclin B1 in mitotic keratoacanthoma cells in UVB-pretreated high-risk mice, but this was not observed in areas of the epidermis away from tumors (Table 3). We were unable to continue these studies with squamous cell carcinomas because of the unavailability of additional cyclin B1 antibody suitable for immunohistochemical studies.

We used the Pearson's correlation coefficient for evaluating the relationship between the percentage of mitotic cells with cyclin B1 in the tumors described in Table 3 and the percentage of mitotic cells with caspase 3 in the same tumors from acetone-treated control mice and mice treated with caffeine (data not shown). We found a statistically significant relationship between the percentage of mitotic cells with cyclin B1 and the percentage of mitotic cells with caspase 3 in individual tumors. In keratoacanthomas from the combined acetone- and caffeine-treated mice, the  $r$  was 0.280 ( $p < 0.01$ ). Our results suggest that an increased level of cyclin B1 in mitotic cells was associated with increased apoptosis in mitotic cells.

In summary, the mechanistic studies reported here suggest that topical applications of caffeine to high-risk mice enhance apoptosis in UVB-induced tumors by decreasing the level of phospho-Chk1 (Ser317), increasing the number of mitotic cells with cyclin B1 and by increasing the number of mitotic cells undergoing apoptosis. The present report provides a mechanistic explanation for our earlier study indicating that topical applications of caffeine to UVB-pretreated high risk mice increases apoptosis in the tumors [7]. The present study provides the first evidence that caffeine administration inhibits the ATR/Chk1 pathway and enhances lethal mitosis *in vivo* within tumors. The results suggest that caffeine-induced inhibition of the ATR/Chk1 pathway may protect animals from cancer by enhancing the removal of proliferating cancer cells during the course of UVB-induced carcinogenesis.

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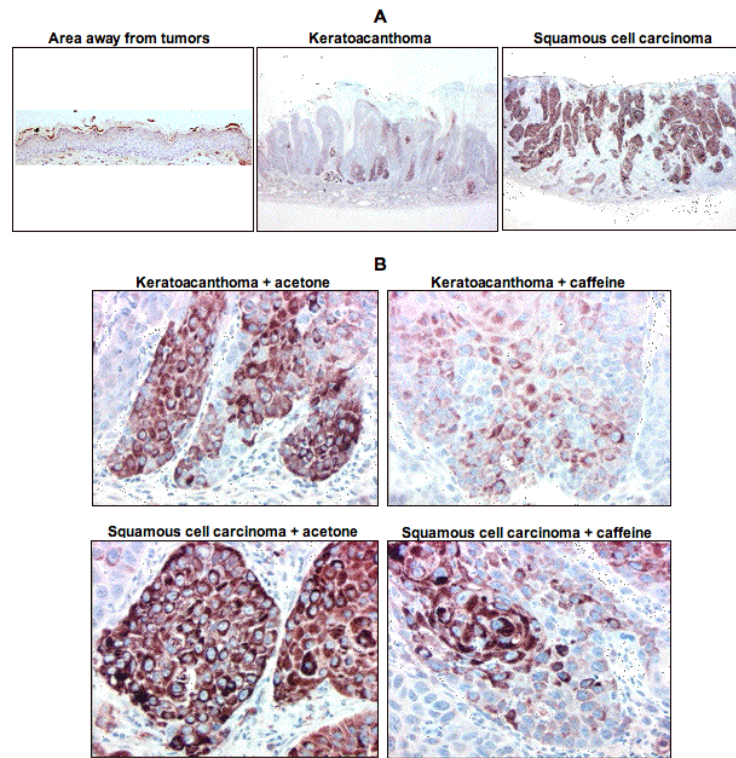
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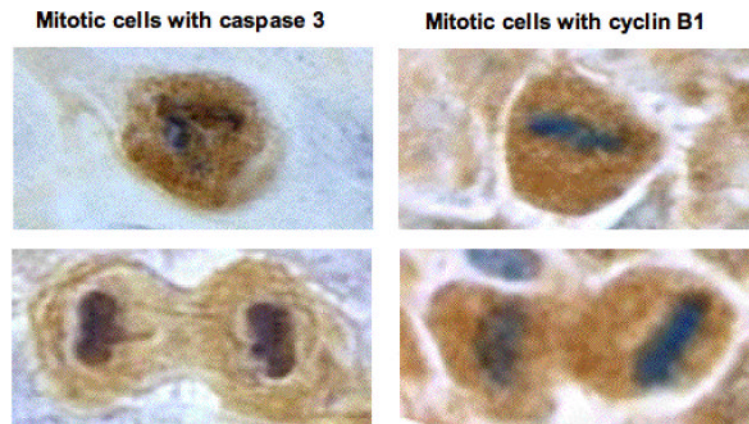




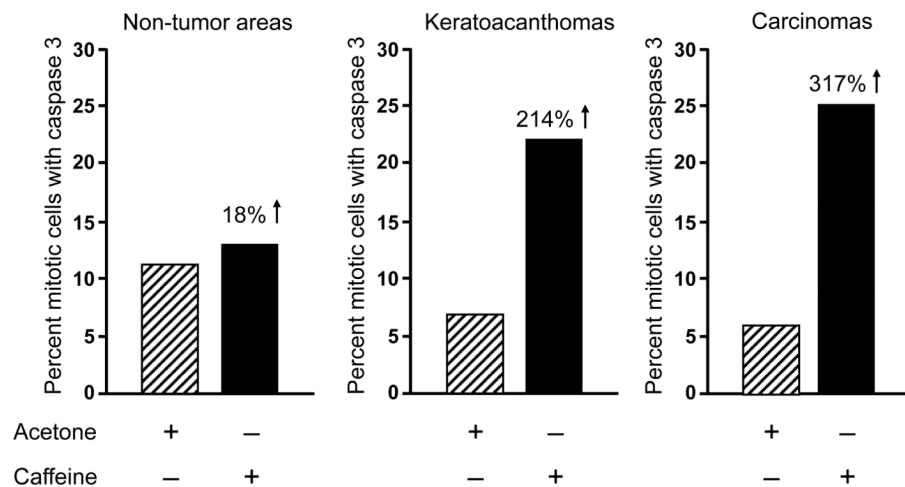
**Fig 1. Effect of topical applications of caffeine to decrease the expression of phospho-Chk1 (Ser317) in UVB-induced skin tumors**

In A, female SKH-1 mice (7-8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks and UVB treatment was stopped. These tumor-free high-risk mice were killed 21 weeks later, phospho-Chk1 (Ser317) was determined immunohistochemically (microscope magnification= 100-fold for epidermal area away from tumors and 40-fold for tumors). Although phospho-Chk1 (Ser317) was observed in tumors, no phospho-Chk1 (Ser317) was observed in 641 normal appearing areas away from tumors (~ 215,000 cells). In B, high-risk mice were treated with 100  $\mu$ l acetone or 6.2  $\mu$ mol caffeine in 100  $\mu$ l acetone once daily 5 days a week for 18 weeks as described in Tables 1 - 3.

Keratoacanthomas or squamous cell carcinomas were stained with an antibody to phospho-Chk1 (Ser317). The inhibitory effect of caffeine administration on the expression of phospho-Chk1 (Ser317) described here is representative of data from 18 mice (acetone group) and 13 mice (caffeine group), respectively (microscope magnification= 400-fold).



**Fig 2. Illustration of mitotic cells with caspase 3 or mitotic cells with cyclin B1 positive staining**  
This figure shows representative mitotic cells with caspase 3 (left) or mitotic cells with cyclin B1 (right) positive staining (microscope magnification= 1000-fold). The tumor sample was from a UVB-pretreated high-risk mouse treated with caffeine for 18 weeks. Similar images were obtained in tumors from control mice.



**Fig 3. Effect of topical applications of caffeine on the percentage of mitotic cells with cyclin B1 or caspase 3 in UVB-induced tumors**

High risk UVB-pretreated mice were treated topically with caffeine for 18 weeks as described in Fig 1 and ref 7. Data for the percent of mitotic cells with caspase 3 in non-tumor areas of the epidermis was obtained from 92,000-123,000 cells, data for the percent of mitotic cells with caspase 3 in keratoacanthomas was obtained from 426,000-882,000 cells, and data for the percent of mitotic cells with caspase 3 in squamous cell carcinomas was obtained from 110,00-555,000 cells as described in Table 2.

Table 1

Inhibitory effect of topical applications of caffeine on the formation of phospho-Chk1 (Ser317) in skin tumors of high-risk mice previously treated with chronic UVB

Treatment	No. of tumors examined	No. of cells examined	Percent tumor cells with caspase 3-positive staining	Percent phospho-Chk1 (Ser317) positive tumors	Percent tumor cells with phospho-Chk1 (Ser317) positive staining	phospho-Chk1 (Ser317) staining (intensity score)
<b>Keratoacanthoma</b>						
Acetone	59	258,000	0.194 ± 0.020	41	5.84 ± 1.43	0.68 ± 0.16
Caffeine	50	176,000	0.469 ± 0.045 <sup>a</sup>	18 <sup>a</sup>	2.09 ± 0.94 <sup>b</sup>	0.18 ± 0.06 <sup>b</sup>
Percent change	-	-	142	-56	-64	-74
<b>Squamous cell Carcinoma</b>						
Acetone	33	555,000	0.196 ± 0.022	64	11.97 ± 3.36	1.49 ± 0.30
Caffeine	10	110,000	0.376 ± 0.056 <sup>a</sup>	40	10.45 ± 4.85	0.40 ± 0.16 <sup>b</sup>
Percent change	-	-	92	-38	-13	-73

Female SKH-1 mice (7-8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks and UVB treatment was stopped. Three weeks later, these tumor-free high-risk mice were randomized and divided into two groups (30 mice per group) and treated topically with 100 µl acetone or with caffeine (6.2 µmoles) in 100 µl acetone once daily 5 days a week for 18 weeks. phospho-Chk1 (Ser317) and caspase 3 positive cells were determined immunohistochemically. Substantially stronger staining for phospho-Chk1 (Ser317) was observed in islands of positive cells in squamous cell carcinomas than in islands of positive cells in keratoacanthomas, and treatment with caffeine decreased the intensity of staining in both types of tumors. The intensity of staining was quantified from all tumor cells using the following intensity scoring system: 0, no to very weak; 1, very weak; 2, weak; 3, moderate; 4, strong; 5, very strong. Each value represents the mean ± SE.

<sup>a</sup> p<0.01

<sup>b</sup> p<0.05.

**Table 2**  
**Stimulatory effects of topical applications of caffeine on the formation of mitotic cells with caspase 3-positive staining in skin tumors of high-risk mice previously treated with chronic UVB**

Treatment	No. of non-tumor areas or tumors examined	No. of cells examined	Percent of cells with mitosis	Percent of cells with both mitosis and caspase 3 positive staining	Percent of mitotic cells with caspase 3 positive staining
<b><u>Non-tumor areas</u></b>					
Acetone	370	123,000	$0.009 \pm 0.001$	$0.001 \pm 0.001$	11
Caffeine	271	92,000	$0.008 \pm 0.001$	$0.001 \pm 0.001$	13
Percent change			-11	0	18
<b><u>Keratoacanthoma</u></b>					
Acetone	202	882,000	$0.351 \pm 0.023$	$0.023 \pm 0.005$	7
Caffeine	121	426,000	$0.316 \pm 0.027$	$0.071 \pm 0.013^a$	22
Percent change			-10	209	214
<b><u>Squamous cell Carcinoma</u></b>					
Acetone	33	555,000	$0.402 \pm 0.046$	$0.026 \pm 0.006$	6
Caffeine	10	110,000	$0.330 \pm 0.033$	$0.081 \pm 0.034^a$	25
Percent change			-18	212	317

Female SKH-1 mice (7-8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks and UVB treatment was stopped. Three weeks later, these tumor-free high risk mice were randomized and divided into two groups (30 mice per group) and treated topically with 100  $\mu$ l acetone or with caffeine (6.2  $\mu$ moles) in 100  $\mu$ l acetone once daily 5 days a week for 18 weeks. Caspase 3 positive cells were determined immunohistochemically. The percentage of mitotic cells with caspase 3 positive staining in the tumors was analyzed. The entire areas of all tumor sections were examined. Non-tumor areas were at least 1 cm away from tumors. Each value represents the mean  $\pm$  SE.

<sup>a</sup> $P < 0.01$ .



**Table 3**  
**Stimulatory effect of topical applications of caffeine on the formation of mitotic cells with cyclin B1-positive staining in skin tumors of high-risk mice previously treated with chronic UVB**

Treatment	No. of non-tumor areas or tumors examined	No. of cells examined	Percent of cells with mitosis	Percent of cells with both mitosis and cyclin B1	Percent of mitotic cells with cyclin B1
<b>Non-tumor areas</b>					
Acetone	70	24,000	0.013 ± 0.001	0	0
Caffeine	63	22,000	0.013 ± 0.001	0	0
Percent change	-	-	0	0	0
<b>Keratoacanthoma</b>					
Acetone	38	73,000	0.300 ± 0.028	0.029 ± 0.003	10
Caffeine	28	69,000	0.280 ± 0.036	0.048 ± 0.007 <sup>a</sup>	17
Percent change	-	-	-7	66	70

Female SKH-1 mice (7-8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks and UVB treatment was stopped. Three weeks later, these tumor-free high risk mice were randomized and divided into two groups (30 mice per group) and treated topically with 100 µl acetone or with caffeine (6.2 µmoles) in 100 µl acetone once daily 5 days a week for 18 weeks. Percent of cells with mitosis or mitotic cells with cyclin B1 positive staining were determined. Each value represents the mean ± S.E.

<sup>a</sup> p<0.01.