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Nicotinamide enhances repair of ultraviolet radiation-induced DNA damage in human keratinocytes and ex vivo skin

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Nicotinamide (vitamin B3) protects from ultraviolet (UV) radiation-induced carcinogenesis in mice and from UV-induced immunosuppression in mice and humans. Recent double-blinded randomized controlled trial 2 studies in heavily sun-damaged individuals have shown that oral nicotinamide significantly reduces premalignant actinic keratoses, and may reduce new non-melanoma skin cancers. Nicotinamide is a precursor of nicotinamide adenine dinucleotide (NAD+), an essential coenzyme in adenosine triphosphate (ATP) production. Previously, we showed that nicotinamide prevents UV-induced ATP decline in HaCaT keratinocytes. Energy-dependent DNA repair is a key determinant of cellular survival after exposure to DNA-damaging agents such as UV radiation. Hence, in this study we investigated whether nicotinamide protection from cellular energy loss influences DNA repair. We treated HaCaT keratinocytes with nicotinamide and exposed them to low-dose solar-simulated UV (ssUV). Excision repair was quantified using an assay of unscheduled DNA synthesis. Nicotinamide increased both the proportion of cells undergoing excision repair and the repair rate in each cell. We then investigated ssUV-induced cyclobutane pyrimidine dimers (CPDs) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxoG) formation and repair by comet assay in keratinocytes and with immunohistochemistry in human skin. Nicotinamide reduced CPDs and 8oxoG in both models and the reduction appeared to be due to enhancement of DNA repair. These results show that nicotinamide enhances two different pathways for repair of UV-induced photodamage, supporting nicotinamide’s potential as an inexpensive, convenient and non-toxic agent for skin cancer chemoprevention.

Introduction

Non-melanoma skin cancers, comprising mostly basal cell carcinomas and squamous cell carcinomas, are the most common malignancies in light-skinned populations, with worldwide skin cancer incidence continuing to increase (1). Ultraviolet (UV) radiation is the primary cause of skin cancer (2). UVB (290–320 nm) damages DNA directly resulting in the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts (6–4PPs). CPDs give rise to UV-signature C to T and CC to TT transition mutations. UVA (320–400 nm) induces CPDs, but also causes singlet oxygen photosensitization-induced DNA photodamages including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxoG) (3). UVB, through less understood mechanisms, has also been shown to induce 8oxoG in murine keratinocytes (4) and primary human epidermal keratinocytes (5). Non-melanoma skin cancer incidence increases with age (6), and age-associated decreases in nucleotide excision repair (NER) of CPDs in in vivo human skin (7) and in oxidative repair of 8oxoG by human 8-oxoguanine-DNA glycosylase 1 (hOGG1) in ex vivo human lymphocytes (8) have been reported. There is some evidence that DNA repair is deficient in patients with basal cell carcinoma (9). Unrepaired CPDs have been found in actinic keratoses and squamous cell carcinomas (10), and recently it has been shown that basal cell carcinomas are deficient in hOGG1 and have a correspondingly high level of unrepaird 8oxoG (11).

Nicotinamide, an amide form of vitamin B3, prevents UV-induced immune suppression and tumor formation in mice (12). In humans, nicotinamide prevents UV-induced immune suppression (13,14) and oral nicotinamide reduced precancerous actinic keratoses by ~30% relative to placebo within 2–4 months in our heavily sun-damaged Australian patients. In these recent Phase 2 studies, numbers of new non-melanoma skin cancers were also significantly reduced in patients randomized to receive nicotinamide (15). Nicotinamide is a precursor of nicotinamide adenine dinucleotide (NAD+), a key coenzyme in cellular metabolism and energy production, and the sole substrate for the DNA repair enzyme poly-ADP-ribose polymerase (PARP) (16,17). Recently, we showed that nicotinamide prevents UV-induced cellular energy loss in human keratinocytes (18). DNA damage is a key trigger of photoinmunosuppression (19), and agents that enhance DNA repair, such as liposomal DNA repair enzymes, have been shown to reduce the immune suppressive effects of UV exposure (20). In this study, we present data showing that nicotinamide increases DNA excision repair activity in HaCaT human keratinocytes and enhances repair of CPDs and 8oxoG following exposure of human keratinocytes and ex vivo human skin to solar-simulated UV (ssUV; UVB + UVA). This enhancement of DNA repair may contribute to nicotinamide’s apparent immune protective and chemopreventive effects against skin cancer.

Materials and methods

HaCaT cells, ex vivo human skin, nicotinamide treatment and ssUV irradiation

The HaCaT human keratocytce cell line was grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Carsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (HyClone, Logan, UT). The concentration of FBS was reduced to 0.5% (vol/vol) 24h before irradiation, in order to minimize the number of cells in replicative DNA synthesis (21). Three healthy volunteers who underwent abdominalplasty (n = 2) and breast reduction (n = 1) gave written informed consent prior to surgery for the use of their skin in the study (approved by the Human Ethics Committee of the University of Sydney according to Declaration of Helsinki principles). The skin was cut into 4 mm² pieces and incubated in RPMI 1640 medium (Invitrogen) supplemented with 10% (vol/vol) FBS and 100 U/ml penicillin, 0.1 mg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen). Nicotinamide (Sigma–Aldrich, St Louis, MO) at a final concentration of 50 μM was added to the media and the cells and skin sections were incubated in their respective media for 24h in a 37°C humidified atmosphere containing 5% CO₂. Semiconfluent cells (70%) and skin pieces were washed twice with phosphate-buffered saline and then irradiated in phosphate-buffered saline with 4 J/cm² ssUV using a 1000W xenon-arc Oriel solar simulator (Newport, Stratford, CT). The ssUV spectrum comprised 9.5% UVB and 90.5% UVA, closely matching the spectrum of natural sunlight (22). UV irradiance was measured before each irradiation using an IL-1700 broadband radiometer (International Light, Newburyport, MA) calibrated against the source with an OL-754 scanning spectroradiometer (Optronic Laboratories, Orlando, FL).

Unscheduled DNA synthesis

Following irradiation, HaCaT cells were further incubated for 45 min at 37°C in 10 μM of BrdU (BD Pharmingen, San Diego, CA) with or without 50 μM nicotinamide. The cells were fixed in cold methanol/glacial acetic acid (3:1) for 20min at 4°C, washed with 100% ethanol and stored at −20°C.

Abbreviations:
8oxoG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ANOVA, analysis of variance; ATP, adenosine triphosphate; BrdU, bromodeoxyuridine; CPDs, cyclobutane pyrimidine dimers; FBS, fetal bovine serum; hOGG1, human 8-oxoguanine-DNA glycosylase 1; NAD+, nicotinamide adenine dinucleotide; NER, nucleotide excision repair; PARP, poly(ADP)-ribose polymerase; ssUV, solar-simulated UV; T4N5, T4-endonuclease V; UDS, unscheduled DNA synthesis; UV, ultraviolet.

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Bromodeoxyuridine (BrdU) immunostaining was performed as described (23) using purified mouse anti-BrdU monoclonal primary antibody (BD Pharmingen) and biotinylated F(ab′)2 fragment of goat anti-mouse secondary antibody (Invitrogen). Alexa-fluor 594-conjugated streptavidin (Invitrogen) was used to visualize primary and secondary antibody bindings. UDS was analyzed by counting punctuated spots in the nuclei using image analysis software (Image-Pro Plus v7.0, Media Cybernetics, Bethesda, MD) and counting the UDS nuclei with punctuated spots, in contrast to the S-phase nuclei, which are uniformly and intensely stained (24). The percentage of UDS and S-phase nuclei was calculated in at least 10 fields (×40 objective) for every treatment group.

Measurement of CPDs and 8oxoG in HaCaT cells by comet assay

Following irradiation in phosphate-buffered saline, HaCaT cells were incubated for 0, 30, 60, 90 and 120 min with or without 50 μM nicotinamide in Dulbecco’s modified Eagle’s medium with 10% FBS at 37°C before detachment from culture flasks for measurement of DNA damage by alkaline gel single cell electrophoresis (comet assay). Further 15 min incubation at 37°C (10 min in 0.02% ethylenediaminetetraacetic acid (wt/vol) (Sigma–Aldrich), 5 min in 0.05% (vol/vol) Trypsin-ethylenediaminetetraacetic acid solution (Invitrogen) were undertook to detach the cells gently, avoiding unwanted DNA damage. Comet assays were performed as we have described previously (25). No enzyme controls were subtracted to calculate the T4N5 or hOGG1-sensitive sites. Three separate experiments were performed for each group (UV; UV + nicotinamide; no UV; no UV + nicotinamide) and for each time point. At least 50 cells were analyzed for each treatment group.

Measurement of CPDs, 8oxoG and hOGG1 in ex vivo human skin by immunohistochemistry

Following ssUV irradiation, skin pieces were further incubated with or without 50 μM nicotinamide for various times in RPMI with 10% FBS as described above. Duplicate skin pieces per time point per treatment group were snap-frozen in OCT (Tissue-Tek, Sakura, Zoeterwoude, Netherland) and stored at −70°C. Four micrometer thick tissue sections were stained using mouse monoclonal anti-thymine dimer (CPD) antibody (clone KTMS5, Kamiya Biomedical, Seattle, WA) at 1:120 dilution for 90 min or mouse monoclonal anti-8-oxoG antibody (clone 2E2; Trevigen, Gaithersburg, MD) at 1:250 dilution for 1 h or rabbit polyclonal anti-hOGG1 antibody (Novus Biologicals, Littleton, CO) at 1:100 dilution for 1 h. For visualization, Dako REAL™ Detection Systems (Dako, Glostrup, Denmark) including biotinylated link antibody (mouse/rabbit), streptavidin conjugated with alkaline phosphatase and a Red chromogen, were used according to manufacturer’s instruction. Quantification of staining (percentage of epidermal area positively stained for CPD and 8oxoG, average intensity for positively stained epidermal area for hOGG1) (26) was performed using ImageJ 1.38x image analysis software (National Institute of Health, Bethesda, MD). CPD and 8oxoG analysis used an intensity threshold above background to calculate percentage staining, allowing us to compare relative photolesion staining in nicotinamide versus control-treated cells. The different method for detection of photolesions, anti-body, compared with excision enzymes in the HaCaT experiments, was used to give greater confidence in the conclusions of the study.

Statistical analysis

In all studies, data were based on at least three independent experiments conducted on at least duplicate samples. Results are expressed as means ± SEM. For time course studies, where four groups were compared in five time points, data were based on at least three independent experiments conducted on at least duplicate samples. Results are expressed as means ± SEM. When only one time point was used in the study, significant differences between corresponding groups (UV versus UV + nicotinamide and DNA damage. Exposure to 4 J/cm2 ssUV induced formation of T4N5-comet assay detected CPDs and abasic sites consistent with the UDS data that nicotinamide alone did not cause DNA damage. Exposure to 4 J/cm2 ssUV induced formation of T4N5-sensitive sites (CPDs and abasic sites). At 45 and 75 min, respectively, after irradiation, there were 19.9 and 38.6% relative reductions in T4N5-tail moment in UV + nicotinamide-treated cells compared with UV-treated cells (Figure 2a). Using repeated measures ANOVA to compare tail moment values over the repair time course, nicotinamide treatment did not cause DNA damage as indicated by a lack of effect on UDS. However, it stimulated a further significant increase in the percentage of UDS in ssUV-irradiated cells (UV, 20.5% versus UV + nicotinamide, 25.2%; P = 0.01), suggesting an enhancement of DNA repair. There was no significant difference in the percentage of S-phase cells in any treatment group showing that this low dose of ssUV was not sufficient to cause cell cycle arrest and that nicotinamide did not affect cell division under these conditions (Figure 1b). The number of grains per nucleus, representing sites of excision repair in each individual cell (27) was also increased by ssUV (no UV, 46.3 grains/nucleus versus UV, 53.7 grains/nucleus; P = 0.0089) (Figure 1c). Nicotinamide further significantly increased the number of grains per nuclei (UV, 53.7 grains/nucleus versus UV + nicotinamide, 62.1 grains/nuclei; P = 0.049), consistent with nicotinamide increasing not only the number of cells undergoing repair of UV-induced DNA damage but also the rate of repair in each cell.

Low-dose ssUV and nicotinamide did not affect HaCaT cell viability

To determine whether the low dose of ssUV (4 J/cm2) used in these studies and incubation with 50 μM nicotinamide affected HaCaT cell survival, cell viability was assessed at 15, 45, 75, 105 and 135 min after ssUV irradiation. There were no differences in viability between groups treated with and without ssUV or nicotinamide at any time points after ssUV irradiation (repeated measures ANOVA). Viability exceeded 85% in all groups at all times (Supplementary Table S1, available at Carcinogenesis Online) indicating that this dose of ssUV was too low to cause substantial cell death and nicotinamide did not influence viability with or without ssUV.

Nicotinamide enhanced repair of CPDs and 8oxoG in HaCaT keratinocytes

Comet assays using T4-endonuclease V (T4N5) and hOGG1 DNA excision enzymes were used to detect CPDs and 8oxoG, respectively, in HaCaT cells. Following 24 h incubation with or without 50 μM nicotinamide, cells were exposed to 4 J/cm2 ssUV. Immediately after irradiation, cells were further incubated with or without nicotinamide for 0, 30, 60, 90 and 120 min. Due to the 15 min additional time taken to detach the cells from culture plates, the comet assay time course was reported as 15, 45, 75, 105 and 135 min following irradiation. A T4N5 comet assay detected CPDs and abasic sites (apurinic or apyrimidinic sites), observed as increased comet length and fluorescence intensity (tail moment). Unirradiated cells with or without nicotinamide did not have a detectable tail moment (Supplementary Figure S3a, available at Carcinogenesis Online), consistent with the UDS data that nicotinamide alone did not cause DNA damage. Exposure to 4 J/cm2 ssUV induced formation of T4N5-sensitive sites (CPDs and abasic sites). At 45 and 75 min, respectively, after irradiation, there were 19.9 and 38.6% relative reductions in T4N5-tail moment in UV + nicotinamide-treated cells compared with UV-treated cells (Figure 2a). Using repeated measures ANOVA to compare tail moment values over the repair time course, nicotinamide was found to significantly enhance the repair of CPDs (P = 0.0001), consistent with the UDS data on nicotinamide stimulation of DNA excision repair. A hOGG1 comet assay measured cleaved 8oxoG and DNA-containing formamidopyrimidine moieties. 8oxoG is a ubiquitous oxidative DNA damage formed by various endogenous and exogenous biochemical processes (28) and was therefore found as expected in unirradiated keratinocytes (Supplementary Figure S3b, available at Carcinogenesis Online and Figure 2b). 8oxoG was significantly increased by 4 J/cm2 ssUV, detected at 15 and 45 min after irradiation. In UV-irradiated cells, a 45.6% relative reduction in 8oxoG at 45 min after irradiation was observed in cells pretreated with...
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At 75 min after ssUV, most 8oxoG had been removed by excision repair. Nicotinamide significantly reduced the number of 8oxoG photolesions over the time course studied ($P < 0.0001$; repeated measures ANOVA), showing that nicotinamide increased repair of this photolesion.

Nicotinamide enhanced repair of CPDs and 8oxoG in ex vivo human skin

Exposure of ex vivo skin to 4 J/cm$^2$ ssUV resulted in significant formation of CPDs and a significant increase in 8oxoG above baseline levels in the epidermis (Figure 3a and b). Nicotinamide alone did not increase levels of either CPDs or 8oxoG in unirradiated skin (repeated measures ANOVA) consistent with previous findings that it did not cause DNA damage. CPDs were first detected at 15 min after irradiation and nicotinamide pretreatment did not affect the initial formation of CPDs in the epidermis (Figure 4a). Nicotinamide caused 26.5 and 34.9% relative reductions in the number of epidermal cells containing detectable CPDs in irradiated epidermis at 45 and 75 min, respectively, and significantly reduced CPD-containing epidermal cells over the time course studied ($P = 0.0496$; repeated measures ANOVA), indicating enhancement of repair, consistent with our other data. Four Joules per square centimeter ssUV induced 8oxoG photolesions significantly above baseline values, reaching a maximum level 45 min after irradiation (Figure 4b). Pretreatment with nicotinamide decreased the number of epidermal cells with detectable 8oxoG to levels equivalent to those in unirradiated epidermis.
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(P = 0.0023; repeated measures ANOVA), showing that nicotinamide also enhances repair of 8oxoG photolesions in human skin.

Nicotinamide enhancement of 8oxoG repair is not mediated by an increase in hOGG1 expression

We investigated whether nicotinamide influenced the expression of the 8oxoG repair enzyme, hOGG1 by protein staining. At 45 min after irradiation, there was no difference in hOGG1 expression in any groups (Supplementary Figures S4 and 5, available at Carcinogenesis Online), suggesting that enhancement of 8oxoG repair by nicotinamide is not due to increased levels of hOGG1.

Discussion

Using the UDS assay in irradiated HaCaT human keratinocytes, we demonstrated an increase in DNA excision repair in cells treated with nicotinamide. UDS is a direct measure of DNA excision repair, a pathway that not only repairs most forms of base damage but also DNA strand breaks and DNA cross links (29). Our ssUV radiation was delivered at a low dose, found to be suberythemal in our previous in vivo studies in humans, and equivalent to about 15 min exposure to noon spring sunlight in Sydney (13). This UV dose thus models routine daily sunlight exposure rather than high dose erythemal recreational exposure. We assessed cell viability after 4 J/cm² ssUV at various time points up to 135 min, the time over which DNA repair was assessed. Cell survival was not affected by the doses of nicotinamide and ssUV used in this study, consistent with our previous findings at similar UV doses (18). The ssUV dose used was too low to reduce cell viability or cause cell cycle arrest as shown by the lack of effect on cells entering division. Therefore, these issues of loss of UV damaged cells or arrest of cell division did not interfere with our assays for detection of DNA repair. This low-dose irradiation, however, resulted in an almost 3-fold increase in the proportion of the cells undergoing UDS within 45 min of irradiation, showing that it did damage DNA and initiate DNA repair. Treatment of HaCaT cells with 50 μM nicotinamide for 24h before irradiation stimulated a further significant 23.3% relative increase in the number of cells undergoing repair. Nicotinamide also increased the rate of DNA excision repair in UV damaged cells as detected by grain numbers within cells, reflecting the amount of incorporated thymidine per cell during excision repair. These findings indicate that nicotinamide stimulates not only the proportion of cells undergoing repair of UV-induced DNA damage but also the repair rate in each cell.
In human lymphocytes treated with UV radiation, 2 mM nicotinamide prevented lowering of NAD⁺ and stimulated a 2-fold increase in UDS compared with control cells treated with UV alone (30). Interestingly, nicotinamide has a more dramatic effect in stimulating UDS in irradiated lymphocytes derived from old mice than in irradiated lymphocytes obtained from young mice, suggesting NAD⁺ level as a critical factor in regulating DNA repair in old mice (31).

Using HaCaT cells and ex vivo human skin, we also investigated the effect of nicotinamide on the two most commonly studied UV-induced DNA photolesions, CPDs and 8oxoG. A single dose of 4 J/cm² ssUV induced formation of CPDs and 8oxoG in both HaCaT cells and human epidermis. There was no reduction with nicotinamide in CPDs detected by either system or 8oxoG photolesions detected by the comet assay at 15 min after irradiation, indicating that nicotinamide did not reduce the formation of these photolesions. This excluded any UV filtering effect of nicotinamide, as we reported previously (13). In HaCaT cells, detectable T4N4 and hOGG1-sensitive sites reached maximum levels at 45 min post ssUV, with >80% of T4N5 and all hOGG1-sensitive sites repaired by 135 and 75 min, respectively. Repair was slower in whole human skin than in HaCaT cells, with a decrease of 60.9% in CPDs and 70.4% in 8oxoG from the maximum levels at 135 min after irradiation. Mouret et al. (32) similarly reported slower repair of DNA photo products in skin biopsies than in cultured primary human keratinocytes and fibroblasts. Delayed formation of CPDs and 8oxoG after UV, as observed in this study, has also been reported previously (33). Peak levels of detected photolesions using the comet assay technique were observed at 45 min after irradiation, probably reflecting changes in the targeted substrate, such as DNA relaxation and increasing accessibility in the context of repair (25,34). Others have observed similar repair time courses to our study for CPDs (25,35,36) and 8oxoG (25,37). Hence, the kinetics of formation and repair of these photolesions, which are likely to vary in different studies depending on UV dose and subsequent damage to repair processes, are similar to previous reported studies with similar UV doses to ours.

Although UDS measures the amount of DNA excision repair, its correlation with specific repair of DNA photolesions is not completely understood. Two enzyme systems were utilized in our in vitro comet assay studies, T4N5 which recognizes CPDs and abasic sites, and hOGG1 which cleaves 8oxoG and DNA-containing formamidopyrimidine moieties. Our data showed that nicotinamide enhances excision repair of both T4N5- and hOGG1-sensitive sites. This specificity issue with the enzymes was addressed, along with confirmation of repair enhancing capacity of nicotinamide in human skin rather than a cell line, by immunostaining of whole human skin with specific anti-thymine dimer (38) and anti-8oxoG monoclonal antibodies (39). Immunostaining of whole human skin also allowed localization of CPDs and 8oxoG photolesions in the epidermis and reduced the potential artefactual DNA damage induced during cell harvesting and lysis during DNA isolation in the comet assay. The use of normal human skin also demonstrates that nicotinamide enhancement of repair occurs in normal keratinocytes in their usual physiological and architectural relationship to other cells. The comet assay is, however, a better quantitative method than immunohistochemistry as used in whole human skin. The combination of the in vitro and ex vivo systems used in these studies gives confidence to our evidence that nicotinamide enhances repair of different UV-induced photolesions and therefore acts on a number of pathways.

It is unclear as to how nicotinamide stimulates an increase in at least two pathways of DNA excision repair after UV irradiation. However, nicotinamide is a primary precursor for the synthesis of NAD, NAD⁺ and NADP⁺ (40). NAD⁺, NADP⁺ and their reduced forms, NADH and NADPH, play major roles in cellular metabolism and energy production. Nicotinamide supplementation increases intracellular NAD⁺ (41) and ATP levels in HaCaT human keratinocytes (18). NAD⁺ is also utilized in ADP-ribosyl transferase reactions by three major classes of enzymes: poly(ADP-ribose) polymerases (PARPs), cADP-ribosyl synthases and sirtuins; thus, linking NAD⁺ metabolism to cellular processes involved in DNA-damage responses, control of gene expression, calcium homeostasis, cell death and aging (16). NAD⁺ is the sole substrate for the DNA repair enzyme PARP, and both NAD⁺ and PARP are essential for effective DNA repair, in particular during damage-excision steps (42). NAD⁺ is an essential co-factor in glycolysis and NAD⁺ depletion inhibits glycolysis and in effect ATP production (43). We found previously that 4 J/cm² ssUV radiation caused significant intracellular ATP depletion within 2 h (18). DNA repair is highly energy dependent. Both nNER and double-strand break repair involves remodeling of the chromatin structure to allow access of DNA repair enzymes. This chromatin modification is highly ATP dependent (44). Our previous studies showed that 50 μM nicotinamide prevented ssUV-induced depletion of intracellular ATP and glycolytic blockade in HaCaT cells (18). It is likely that the nicotinamide stimulation of DNA excision repair shown in this study is mediated by its ability to prevent UV-induced ATP decline, making higher amounts of ATP available for DNA repair and other cellular processes. Hence, our study also highlights the importance of cellular energy in the DNA repair process.

We found that nicotinamide enhances DNA repair after UV radiation. This may be a key mechanism of nicotinamide’s demonstrated protective effects against photoimmunosuppression and skin carcinogenesis. Nicotinamide is non-toxic (45), inexpensive and already widely available. It is a promising agent for skin cancer chemoprevention. Phase 3 clinical trials of its effectiveness are now needed.

**Supplementary material**

Supplementary Figures S1–S4 and Table 1 can be found at http://carcin.oxfordjournals.org/

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