The Use of a 3895 bp Mitochondrial DNA Deletion as a Marker for Sunlight Exposure in Human Skin

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Previous work has examined the use of mitochondrial DNA (mtDNA) damage as a biomarker of cumulative sun exposure in human skin. These studies have simply compared mtDNA damage between sun-protected and sun exposed skin. This approach is limited because non-melanoma skin cancer (NMSC) is predominantly formed on body sites which are 'usually' sun exposed as opposed to sites which are 'occasionally' sun exposed and as such they differ in their cumulative ultraviolet (UV) exposure. This study addresses this limitation by investigating the frequency of occurrence of a rarely reported 3895 bp mtDNA deletion in 104 age-matched human skin samples taken from different sun-exposed body sites. There was a significant increase in the deletion frequency with increasing UV exposure ($p < 0.0001$). Furthermore there was a significantly greater deletion frequency in 'usually' sun exposed compared with 'occasionally' sun-exposed body sites in both the dermis ($p = 0.0018$) and epidermis ($p < 0.0001$). Investigation of the 3895 bp deletion in the same NMSC samples used in a previous study of the 4977 bp common deletion, showed a greater frequency of the 3895 bp deletion (8/10 *vs* 4/10, respectively). Additionally, we have linked the 3895 bp deletion with the UVR component of sunlight by inducing the deletion *in vitro* with repetitive sub-lethal doses of a UVA + UVB light source.

Key words: biomarker/DNA deletion/mitochondrial DNA/skin cancer/ultraviolet radiation
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The incidence of non-melanoma skin cancer (NMSC) is increasing in populations of European origin (Severi and English, 2004), e,g., 1 million new cases diagnosed each year in the USA (Wesson and Silverberg, 2003) and 65,000 in the UK (figures provided by Cancer Research, UK). NMSC accounts for around 90% of skin cancers and consists of basal cell and squamous cell carcinomas (BCC and SCC, respectively). BCC are the commonest form of NMSC and arise predominantly from the basal keratinocytes of the epidermis but also from cells in hair follicles and sebaceous glands. They are locally invasive but rarely metastasize. SCC are also derived from basal keratinocytes; however, in contrast to BCC, SCC may metastasize. Compared with BCC, SCC shows the greatest increase with age and is concentrated in the elderly (Severi and English, 2004). The relative density of NMSC is highest on body sites "usually" exposed to the sun when outdoors such as scalp, face, neck, and ears as defined by Armstrong (2004). SCC, however, differs appreciably from BCC in having a much lower density on body sites which are "occasionally" exposed to the sun such as shoulders, back, and chest as defined by Armstrong (2004).

Therefore, the major determinant of NMSC is the ultraviolet radiation (UVR) component of sunlight that induces DNA damage. Importantly it is both the pattern (more continuous *vs* intermittent) and the cumulative amount of sun exposure that influences the development of NMSC (Armstrong and Kricker, 2001). To determine a reliable marker of cumulative UVR exposure in human skin, our research group and others have examined the novel idea of using mitochondrial DNA (mtDNA), rather than nuclear DNA, as a biomarker of UV-induced DNA damage (Pang *et al*, 1994; Berneburg *et al*, 1997; Birch-Machin *et al*, 1998; Birch-Machin, 2000). Compared with mutation screening of nuclear DNA genes such as p53, there are certain advantages of studying mtDNA damage in sun-exposed skin. First, although there is evidence in mitochondria for base excision repair of oxidative damage, there is no evidence of nuclear excision repair for the repair of DNA photoproducts (e.g., cyclobutane pyrimidine dimers) in mtDNA (LeDoux *et al*, 1993; Croteau and Bohr, 1997; Pascucci *et al*, 1997; Sawyer and Van Houten, 1999). Second, each cell can contain up to several thousand copies of the mtDNA genome and mitochondria can therefore tolerate very high levels (up to 90%) of damaged mtDNA through complementation of the remaining wild-type (Chomyn *et al*, 1992; Sciacco *et al*, 1994). Together, these factors lead to accumulation of photodamage in mtDNA without compromising cell function.

The use of mtDNA damage as a biomarker for cumulative sun exposure in human skin is a relatively new field of research and previous work has simply compared mtDNA damage to distinguish between sun-protected and sun-exposed skin (Pang *et al*, 1994; Berneburg *et al*, 1997;

Abbreviations: BCC, basal cell carcinoma; mtDNA, mitochondrial DNA; NMSC, non-melanoma skin cancer; SCC, squamous cell carcinoma; UV, ultraviolet; UVR, ultraviolet radiation

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Birch-Machin *et al*, 1998). This approach is limited because NMSC is predominantly formed on body sites which are "usually" exposed to the sun when outdoors as opposed to sites that are "occasionally" exposed to the sun (Armstrong, 2004). In an attempt to address this limitation, the present study shows that the frequency of occurrence of a rarely reported 3895 bp mtDNA deletion (only previously described in diseased muscle (Moraes *et al*, 1992)) is significantly different between body sites that are "usually" *versus* "occasionally" exposed to the sun. In addition, we have furthered the link between the etiology of the 3895 bp deletion with the UVR component of sunlight by inducing the 3895 bp deletion *in vitro* with repetitive sub-lethal doses of a UVA + UVB light source.

Results and Discussion

Confirming the identity of the 3895 bp deletion We reanalyzed in greater detail the mtDNA deletion spectrum of NMSC and sun-exposed skin from a previous study (Durham *et al*, 2003) and found that many samples harbored a deletion approximately 4 kb in size. Following a search of the MITOMAP (Mitomap, 2004) database, we postulated that the identity of this deletion may be the 3895 bp species that has been reported in the minor arc spanning nucleotides 547–4443. This deletion has previously been associated with Kearns Sayre Syndrome and Chronic Progressive External Opthalmoplegia (Moraes *et al*, 1995). To confirm the identity of the deletion, we designed a deletion specific PCR assay (see methods). The 375 bp product from this PCR was sequenced to confirm that it contained the deletion-junction sequence that is characteristic of the 3895 bp deletion, namely 5′ CTAACC$^{536\ bp/4430\ bp}$ccatacccgaa$^{548\ bp/4442}$ AATGTT 3′. Characteristically, this sequence contained only one of the two 12 bp repeats that flank the 3895 bp deletion in wild-type mtDNA (lower case letters).

A comparison of the frequency of the 3895 bp deletion in body sites which are usually sun exposed *versus* those which are occasionally exposed As the 3895 bp deletion was originally observed in NMSC samples taken from sun-exposed sites, we addressed the question of whether the frequency of the deletion is a marker of increasing cumulative sun exposure. Using the deletion-specific PCR assay (see Materials and Methods), we investigated its frequency of occurrence in 104 age-matched, split human skin samples taken from usually, occasionally and rarely sun-exposed body sites. There was a significant increase in the deletion frequency with increasing UV exposure in both the epidermis ($p < 0.0001$, $\chi^2 = 31.36$, 2 df; Pearson's χ^2 test) and dermis ($p < 0.0001$, $\chi^2 = 28.68$, 2 df) (Fig 1). Importantly, there was a significantly greater deletion frequency in body sites that are "usually" sun-exposed compared to those which are "occasionally" exposed in both the dermis ($p = 0.0018$, $\chi^2 = 9.72$, odds ratio 8.5; χ^2 test) and epidermis ($p < 0.0001$, $\chi^2 = 17.53$, odds ratio 40) (Fig 1*b*). The deletion was not detected in the body sites that were "rarely" sun-exposed (Fig 1*b*). As the mean ages, sex ratios and tumor type from which the perilesional skin was

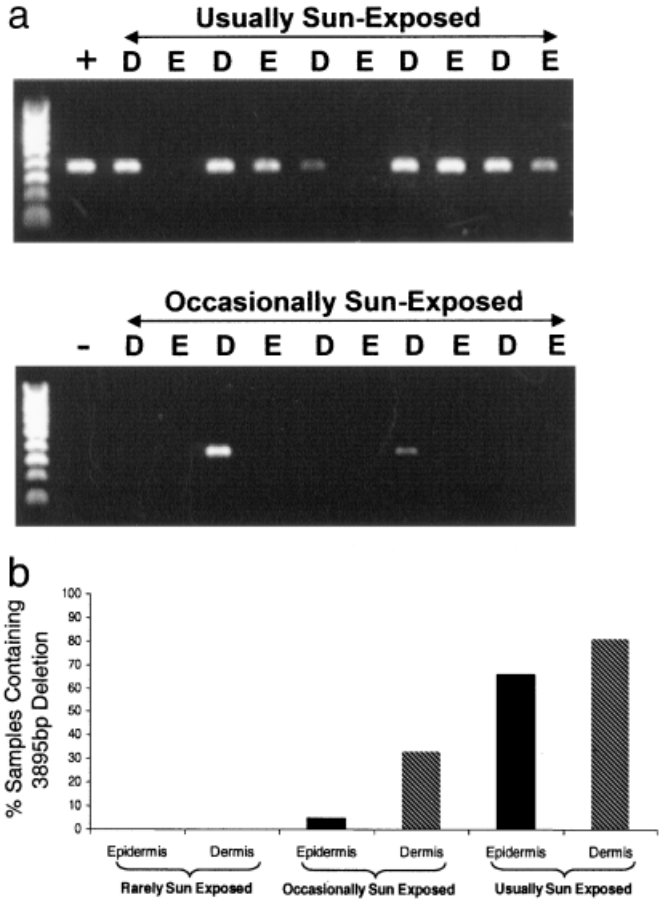

Figure 1
An increased frequency of occurrence of the 3895 bp deletion is observed with increasing sun exposure. (*a*) A representative ethidium bromide stained, agarose gel showing a greater frequency of the 3895 bp deletion in body sites that are usually exposed (*upper panel*) to the sun when outdoors as opposed to those which are occasionally sun-exposed (*lower panel*). D, dermis and E, epidermis. The positive control represents a sample with the 3895 bp deletion that has been confirmed by sequencing. Lane 1 in both panels represents molecular weight markers (Hyperladder IV—range 1000–100 bp, Bioline, London, UK). The same amount of template DNA was added to each PCR reaction. (*b*) The histogram shows the frequency of the 3895 bp deletion in 104 split skin samples taken from different sun-exposed body sites.

taken were very similar between the usually sun-exposed and occasionally exposed groups (see Materials and Methods), our findings are unlikely to be confounded by these factors. In addition, there was no statistical difference in the mean age values of those samples that harbored (i.e., mean $= 66.95 \pm 2.84$) and did not harbor (mean $= 65.81 \pm 3.47$) the 3895 bp deletion ($p = 0.80$, *t* test (Welch correction)).

The 3895 bp mtDNA deletion in NMSC Investigation of the 3895 bp deletion in the same NMSC samples, which were used in a previous study of the 4977 bp common deletion (Durham *et al*, 2003), showed a comparatively greater frequency of occurrence of the 3895 deletion (8/10 *vs* 4/10, respectively), although this difference was not statistically significant. As these tumors were excised from body sites that are usually exposed to the sun, it is interesting to speculate that the 3895 bp deletion may be a more sensitive marker of cumulative sun exposure than the common deletion.

Historically, it has been assumed that the minor arc region of the mitochondrial genome that contains the 3895 bp deletion does not harbor as many deletions as the major arc that contains the common deletion (Wei *et al*, 1996; Mitomap, 2004). As a result, the majority of previous studies have tended to focus on the spectrum of deletions in the major arc region. It could be due to this "research bias" that the 3895 bp deletion has a low reported incidence in the general literature. Alternatively, the 3895 bp deletion may naturally occur at currently undetectable levels in normal tissues, which is then enriched in the skin through exposure to UV.

Absence of the 3895 bp mtDNA deletion in blood The 3895 bp deletion has only previously been reported in diseased muscle (Moraes *et al*, 1992). Apart from the present study in skin, the frequency of occurrence in other tissues is unknown. We therefore investigated the deletion frequency in blood, by performing the deletion-specific PCR on 16 blood samples taken from patients of a similar age group to those of the skin samples. None of the blood samples were shown to harbor the deletion (data not shown).

Generation of the 3895 bp deletion in cultured HaCaT cells by repetitive UV irradiation To assess the causal relationship between the cumulative amount of sun exposure and the frequency of occurrence of the 3895 bp deletion, it was necessary to investigate the effect of sunlight *in vitro*. As sunlight contains both UVA and UVB, an helarium lamp (Diffey, 2002) was used to provide an extensive series of repetitive sub-lethal UVR dose regimes in an attempt to generate the 3895 bp deletion in a human epidermal-derived (HaCaT) cell line. The optimal UVR repetitive dose strategy was one in which the deletion was generated without a significant degree of cell death. Using a radioactive PCR-based assay, we demonstrated that the first signs of induction of the 3895 bp deletion in adherent cells was observed following 17 alternate daily doses of 0.5 J per cm^2 (i.e., 1 SED) of UVR (Fig 2). Furthermore, as sub-lethal UVR doses were used, the level of the deletion was maintained in the cell line following two subsequent UV doses, a property that is important if the 3895 bp can be used as a putative cumulative biomarker of sun exposure in human skin. This is of interest given the very recent findings of Berneburg *et al* (2004) (published during manuscript revision), who have demonstrated *in vivo* that the UVA-induced common deletion may be present 16 months following cessation of irradiation.

Our observations described above are important for several reasons. Firstly, our study has used a UVR source emitting both UVA and UVB thereby representing more closely a solar-simulated UVR source than those previous studies which generated the common deletion using UVA alone (Berneburg *et al*, 1999; Koch *et al*, 2001). Secondly, this is the first time a deletion other than the 4977 bp common deletion has been generated by repetitive doses of UVR. Furthermore, in contrast to the Berneburg study that utilized fibroblasts, our experiments have been performed on a cell line derived from keratinocytes and it is this cell type that gives rise to NMSC.

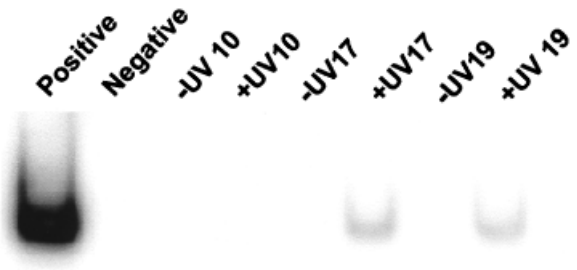

Figure 2
The helarium lamp (UVA/UVB) induces the 3895 bp deletion in HaCaT cells after 17 doses of 0.5 J per cm^2 UVR. HaCaT cells were irradiated with 0.5 J per cm^2 (i.e., 1 SED) of UVA/UVB every other day for a total of 19 doses. Total cellular DNA was extracted from adherent cells and 100 ng was subjected to PCR to amplify the 3895 bp deletion. The first signs of a UV-inducible increase of the 3895 bp deletion was observed following 17 repetitive doses of UVR. The positive control is DNA from a tumor sample harboring the 3895 bp deletion, whereas the negative control does not contain DNA.

Functional significance The regions that are deleted in the 3895 bp deletion are from the mtTF1 binding site in the D-loop to tRNA methionine. Deleted genes include 12s rRNA, 16s rRNA, ND1, and also the promoters for transcription of both the H and L strands. A certain threshold of wild-type:deleted mtDNA must be achieved before an impairment of mitochondrial respiratory function is observed (Sciacco *et al*, 1994). For protein-coding mtDNA genes, such as those removed by the 3895 bp deletion, the threshold value for mitochondrial respiratory chain dysfunction is around 65% and above (Hayashi *et al*, 1991; Chomyn *et al*, 1992). For example, our previous work has shown that human skin samples harboring $<25\%$ of the 4977 bp mtDNA common deletion, do not exhibit a deficiency of mitochondrial function as determined by dual histochemical staining of cytochrome oxidase and succinate dehydrogenase activities.[2] Since there is no functional histochemical stain for the 3895 bp deletion, we attempted to quantify the deletion in our patient samples using Southern analysis. In the presence of appropriate controls, this analysis failed to detect the presence of the 3895 bp deletion thereby suggesting that the levels of the deletion are below 2%–5% (results not shown). Therefore, based on our previous work with the common deletion, the levels of the 3895 bp deletion in our patient samples are unlikely to cause any functional effect across the entire dermis or epidermis, although small focal effects cannot be ruled out.

Putative mechanism It has previously been suggested that the mechanism for the generation of the common deletion involves intragenomic recombination event via slipped-strand mispairing and may occur at the 13 bp repeat DNA sequences flanking the common deletion (Schon *et al*, 1989; Shoffner *et al*, 1989; Mita *et al*, 1990; Degoul *et al*, 1991). As the 3895 bp deletion is flanked by 12 bp repeats, its generation may occur by a similar mechanism. The mechanism for the generation of the common deletion proposes that the 13 bp repeats are susceptible to DNA bending thereby al-

[2]Durham S, Betts J, Birch-machin, M. Histochemical and mutational analysis of mitochondrial function and DNA in tumours and matched normal human skin. *Br J Dermatol* 733, 2002 (abstr).

lowing a small region or "bubble" of single-stranded DNA to open (Schon *et al*, 1989). Our results suggest that UVR may be a contributing factor in the generation of the 3895 bp deletion. The mechanism for this may occur by directly or indirectly affecting the structurally labile sites in the 12 bp repeats through opening a "bubble" of single-stranded DNA that would enhance the recombination event.

Conclusion

In summary, due to limitations by our own previous studies and others, we have shown that the frequency of a rarely reported 3895 bp-mtDNA is significantly different between body sites that are "usually" *versus* "occasionally" exposed to the sun when outdoors. Investigation of the 3895 bp deletion in the same NMSC samples used in a previous study of the 4977 bp common deletion showed a comparatively greater frequency of occurrence of the 3895 bp deletion. In addition, we have furthered the link between the etiology of the 3895 bp deletion with the UVR component of sunlight by inducing the 3895 bp deletion *in vitro* with repetitive sub-lethal doses of a UVA + UVB light source. The frequency of the 3895 bp deletion in human skin provides a potential biomarker for cumulative UV exposure in human skin and may in turn provide an early detection tool for NMSC development as well as providing a method of monitoring long-term safety of clinical UV phototherapy regimes.

Materials and Methods

Patient samples Clinically normal perilesional skin from body sites that are "usually" exposed to the sun when outdoors (such as scalp, face, neck and ears) (epidermis n = 21, dermis n = 21, mean age ± SEM = 69.4 ± 2.6) and body sites that are "occasionally" exposed to the sun (shoulders, back and chest) (epidermis n = 21, dermis n = 21, mean age ± SEM = 63.1 ± 3.6) were taken with informed consent from 42 NMSC patients attending the skin cancer excision clinic at the Royal Victoria Infirmary, Newcastle, UK. There are no significant age differences between the usually and occasionally sun-exposed groups ($p = 0.158$: two-tailed *t* test (Welch correction)). In addition, of the 42 patients, the percentage of females:males were almost the same (i.e., 52%:48%, respectively) as well as the percentage of BCC and SCC, with 57% of the patients having a BCC. Normal skin samples from body sites that are rarely exposed to the sun (such as buttock and heel) were taken from previously obtained postmortem samples (epidermis n = 10, dermis n = 10, mean age = 73 y). Epidermis and dermis were separated using 0.25% dispase at 4°C overnight (Durham *et al*, 2003) and DNA was extracted using a Qiagen, (Qiagen Ltd, Crawley, West Sussex, UK) DNeasy tissue extraction kit. Epidermal tumors, BCC (n = 5), SCC (n = 5) were obtained from patients attending for cancer excision. None of the patients used for this study had a mtDNA defect. This study was performed with full approval of the host institution (University of Newcastle) and in complete adherance to the Helsinki Principles.

UV irradiation of HaCaT cells A spontaneously immortalized keratinocyte cell line (HaCaT) (Boukamp *et al*, 1988) was grown in Dulbecco's modified Eagle's medium containing, 10% fetal bovine serum 5 IU per mL penicillin and 5 g per mL streptomycin. The cells were grown to 70%–90% confluency in a 9 cm diameter tissue culture-treated Petri dishes, washed in PBS and then irradiated every alternate day with a sub-lethal dose (0.5 J per cm^2 which is equivalent to 1 SED) of UVR using an helarium 40 W lamp (Wolff B1.01, 290–400 nm, peak emission at 325 nm) (Helarium, Cosmetico, Stuttgart, Germany). At appropriate time points, total cellular DNA was extracted from the adherent cells using the Qiagen, DNeasy tissue extraction kit.

PCR analysis The PCR was carried out in a 25 μL reaction containing 200 ng genomic DNA, 600 nM of each primer, 250 μM dNTPs, 0.6 U per reaction Amplitaq Gold DNA polymerase (Applied Biosystems Warrington, Cheshire, UK), GeneAmp buffer (containing, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl and 0.01% (wt/vol) gelatin). The PCR primers used were L404 (5′ CTT TTG GCG GTA TGC ACT TT 3′) (404–423 nt) and H4676 (5′ GAT TAT GGA TGC GGT TGC TT 3′) (4676—4657 nt). Primers L404 and H4676 were designed to anneal outside the 3895 bp deletion. During DNA amplification the short (30 s) polymerase extension time did not permit amplification of wild-type PCR products, allowing only amplification of the smaller 375 bp product which represents the deleted mtDNA species. The PCR conditions were 94°C for 10 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension of 7 min at 72°C. Amplification products were visualized in a 1% agarose gel stained with ethidium bromide (0.25 μg per mL).

DNA sequence analysis The 375 bp PCR product was gel excised and purified using QIAquick gel extraction kit (Qiagen Ltd., Crawley, West Sussex, UK) and cloned into a pCR4-TOPO vector using a TOPOTA Cloning kit (Invitrogen, Paisley, Scotland, UK). To confirm the identity of the 375 bp PCR product, the DNA was sequenced using automated DNA sequencing (MWG Biotech, Ebersberg, Germany).

Radioactive PCR analysis To detect low levels of the deletion generated by UVR, the PCR was carried out as described above but with addition of 3 μCi of [α-^{32}P]-dCTP (Amersham, Buckinghamshire, UK). The PCR products were then electrophoresed through a 6% non-denaturing polyacrylamide gel and exposed to a phosphorimage screen for approximately 24 h. The radioactive PCR fragments were scanned and visualized by a Phosphorimager, using the ImageQuant software (Molecular Dynamics, Chesham, UK).

Statistical analysis Statistical analyses were performed using StatCalc (Epi-info. CDC, Alberta, Georgia) employing χ^2, Pearson's χ^2, Fisher's exact and paired *t* tests.

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