Mitochondrial DNA Deletions Serve as Biomarkers of Aging in the Skin, but Are Typically Absent in Nonmelanoma Skin Cancers

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The potential role of mitochondrial DNA (mtDNA) deletions in nonmelanoma skin cancer (NMSC) and in cutaneous photoaging was explored using a genetic approach. Tumors and photodamaged tumor-free "margin" skin were obtained from NMSC patients undergoing excision and the mtDNA from these specimens was screened for the presence of deletions using long extension PCR. mtDNA deletions were abundant in margin tissue specimens from older patients and their number correlated with the patient age. There was a statistically significant difference between the number of mtDNA deletions in tumors and margins. Fewer deletions were detected in the tumors than the margins and the tumors often had no deletions, implying a potential selection for full-length mtDNA or perhaps a protective role for mtDNA deletions in the process of tumorigenesis. The observed mtDNA deletions from skin were often unreported (19 of 21 deletions), but typically shared structural features with mtDNA deletions reported in other tissues. Some mtDNA deletions were detected from the skin of multiple individuals, including 3,715 and 6,278-base pair (bp) deletions, whose frequencies approached that of the previously well-characterized 4977-bp "common" deletion. These data support the use of mtDNA mutations as biomarkers of photoaging in the skin.

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INTRODUCTION

Mitochondria have well-recognized roles both in the generation of cellular energy and as mediators of cellular events such as apoptosis. There is emerging evidence for mitochondrial DNA (mtDNA) changes in the complex processes of cellular aging and neoplasia (Wallace, 1999; Copeland *et al*., 2002; Eng *et al*., 2003). The human mtDNA is a maternally inherited, circular, double-stranded DNA of 16,569 base pairs (bp), encoding 22 tRNAs, two rRNAs, and 13 polypeptides, all of which are subunits of respiratory enzyme complexes in the electron transport chain (Giles *et al*., 1980; Anderson *et al*., 1981, 1999). The remainder of the proteins that function in the mitochondrion are encoded in the nucleus and imported into the mitochondrion. As the mitochondria contain multiple copies of the mtDNA and cells may contain thousands of mitochondria, newly acquired somatic mutations are heteroplasmic in nature. However, replicative segregation may allow mutant mtDNA molecules in some cells to become prominent, or even to become the exclusive mtDNA in the cell, a condition known as homoplasmy.

The mitochondrion serves as the major site for production of adenosine triphosphate through the process of oxidative phosphorylation. Reactive oxygen species (ROS), natural byproducts of this pathway, can damage lipids, proteins, and DNA (Shigenaga *et al*., 1994; Richter, 1995). mtDNA has a high mutation rate due to its lack of histones, decreased capacity for repair, and close proximity to the site of ROS formation (Wallace *et al*., 1992). Imbalances between oxidative stress and free radical scavenging enzymes have been suggested as the underlying causes of most of the mtDNA damage (Shoffner *et al*., 1989; Lu *et al*., 1999; Shin *et al*., 2005). Recent studies have implicated singlet oxygen as a key intermediate in the UVA-induced generation of the 4,977 bp ''common'' mtDNA deletion *in vitro* (Berneburg *et al*., 1999) and that supplementation with the adenosine triphosphate-producing precursor creatine can prevent the 4,977 bp ''common'' deletion from becoming prominent and exerting its effects on adenosine triphosphate synthesis and oxygen consumption (Berneburg *et al*., 2005). A role for

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Abbreviations: BCC, basal cell carcinoma; mtDNA, mitochondrial DNA; NMSC, nonmelanoma skin cancer; ROS, reactive oxygen species

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mutations in mitochondrial proteins has been suggested by many recent studies that found a high frequency of mtDNA mutations in a variety of cancers, including colon, bladder, and lung (Polyak *et al.*, 1998; Fliss *et al.*, 2000).

In contrast to other tissues, the skin is subject to both chronological aging as well as environmental insult in the form of UVR. mtDNA mutations have been found primarily in sun-exposed skin over sun-protected skin (Pang *et al.*, 1994; Yang *et al.*, 1994, 1995; Berneburg *et al.*, 1997, 1999, 2004; Birch-Machin *et al.*, 1998; Ray *et al.*, 2000; Koch *et al.*, 2001; Harbottle *et al.*, 2004; Krishnan *et al.*, 2004). The most common mutation found in aging tissues is the 4,977-bp ''common'' deletion (Cortopassi and Arnheim, 1990; Ikebe *et al.*, 1990; Cortopassi *et al.*, 1992; Wallace, 1992; Sciacco *et al.*, 1994; Brierley *et al.*, 1998; Nagley and Wei, 1998). Although this mutation has been found in numerous tissues that are not susceptible to UVR, its presence in the skin has primarily been associated with sun exposure. In fact, the common deletion has been shown to be inducible both *in vitro* and *in vivo* in human skin and is thought to occur as a result of mtDNA damage mediated through singlet oxygen (Berneburg *et al.*, 1999, 2004). Additionally, the 4,977-bp ''common'' deletion has been proposed to be a biomarker of photo-aged skin because the level of heteroplasmy in the sun-exposed skin increases with age, while such levels are not increased in sun-protected skin (Yang *et al.*, 1994, 1995; Birch-Machin *et al.*, 1998).

mtDNA point mutations have now been identified in many types of tumors (Polyak *et al.*, 1998; Copeland *et al.*, 2002). A number of hypotheses have been proposed to explain this phenomenon. A possible explanation for mutant mtDNA in tumor cells is that mutant mtDNAs might alter mitochondrially mediated apoptotic pathways to escape cell death (Penta *et al.*, 2001). Alternatively, the mtDNA changes may endow the tumor cell with a selective growth advantage directly or combine with acquired nuclear encoded mutations to provide an advantage (Flury *et al.*, 1976; Torroni *et al.*, 1990; Penta *et al.*, 2001). Perhaps the mutant mtDNAs produce protein changes resulting in the liberation of slightly increased levels of ROS. Low levels of increased ROS have been proposed to be mitogenic in contrast to high levels of ROS that are toxic (Toyokuni *et al.*, 1995; Wei, 1998; Davies, 1999; Penta *et al.*, 2001). While various mutations have been associated with photo-aging and neoplasia, it is unclear whether a characteristic signature of mtDNA mutations exists in these conditions.

We sought to characterize mtDNA changes in the photoaged skin and tumors from patients with nonmelanoma skin cancer (NMSC). Our objective was to determine if NMSC has a distinct mtDNA deletion signature. Numerous mtDNA deletions were detected from photoaged, tumor-free skin of multiple individuals including novel 3,715- and 6,278-bp deletions and the 4,977-bp ''common'' deletion, and the number of mtDNA deletions observed in the surgical margins correlated with the patient age. In contrast, tumors contained fewer mtDNA deletions than the margins, suggesting a potential selection for full-length mtDNA in NMSC formation, or perhaps a protective role of mtDNA deletions in the formation of NMSC. Together, these data suggest that mtDNA mutations may potentially be used as biomarkers of photoaging in the skin, but not as characteristic mutation signatures of NMSC itself.

RESULTS

Long-extension PCR reveals different mtDNA deletion patterns in tumors *versus* margins

To determine whether specific deletions provide a signature for tumors, we isolated DNA from both NMSC tumors and tumor-free photo-aged ''margin'' skin from patients. In all, 98% of the mitochondrial genome was amplified using primers oriented in opposite directions in the cytochrome *b* gene using long-extension PCR (Materials and Methods). These PCR products were resolved by field inversion gel electrophoresis to determine whether deleted mtDNA species are present (Figure 1a). mtDNA was amplified from tumors (T) and margins (M) from patients with basal cell carcinoma (BCC) and squamous cell carcinoma of various ages or from a 30-year-old control patient (C) with no history of skin cancer. Full-length mtDNA is indicated by the amplification of a 16.3-kb product, and is the only species observed in the control patient. mtDNAs harboring deletions are indicated by the presence of shorter fragments. While it appears that tumors contain predominantly or exclusively full-length mtDNA, margin samples, on the other hand, contain readily detectable mtDNA deletions (Figure 1a). Older patients had more mtDNA deletions, and sometimes deleted mtDNAs were the only species detected in this assay. The number of deleted mtDNA species strongly correlated with patient age in margins (Figure 1b; Spearman rank correlation coefficient $\rho = 0.93$, $P = 0.003$) but not in tumors (Figure 1b; $\rho = 0.37$, $P = 0.33$). Furthermore, a comparison of the number of deleted mtDNA species present in tumor versus margin samples was statistically significant ($P = 0.009$) using a nonparamentric Wilcoxon signed-rank test.

Characteristics of mtDNA deletions in photoaged skin

To determine the breakpoints of the deletions identified in Figure 1a, various primer pairs were used to amplify all regions of the mtDNA. In this experiment, the primers were designed to be distant, while the extension times were short. (Parameters and primer sequences are given in Materials and Methods and Table S1, respectively.) In this way, only mtDNAs containing deletions will be amplified. Such PCR products were resolved by gel electrophoresis, extracted, and sequenced. Table 1 summarizes the deletions identified. While the 4,977-bp ''common'' deletion was detected frequently, a number of novel deletions were also identified, whose sizes range from 591 to 8,257 bp. Most deletions had 3–15 bp direct or indirect repeats. The majority of the deletions were in the major arc of the mtDNA, the region between the origins of replication of the two strands. Finally, all mutations caused deletions of multiple genes. The deletions identified using this method are presented in Table 1 and Figure S1.

Figure 2 shows the sequences of the breakpoints of two previously undescribed deletions each observed in the

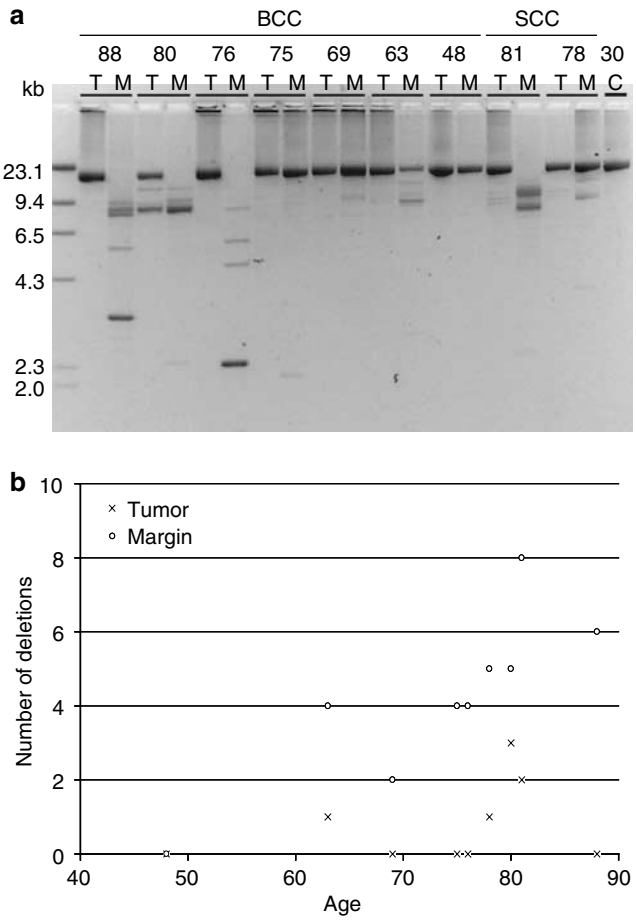

Figure 1. (a) Resolution of deleted mtDNA species. mtDNA isolated from the representative tumor (T) and margin (M) was amplified and resolved. An inverted image of a 0.8% agarose gel is shown. The age of the patient from whom the tumor and margin samples were isolated is indicated above the T and M. The first seven patients had BCC and the last two had squamous cell carcinoma. DNA from a 30-year-old control patient (C) is shown at the right. A molecular weight marker is shown on the left; sizes of marker fragments are given in kb. Full-length mtDNA is indicated by the amplification of a 16.3-kb product (the only species observed in the control patient). mtDNAs harboring deletions are indicated by the presence of shorter fragments. (**b**) The number of deleted mtDNA species is plotted versus age for the tumor and margin samples in (**a**). The Wilcoxon signed-rank test was used to compare non-normally distributed variables in paired samples.

margins of multiple patients. The 6,278-bp deletion (panel A) occurs at an 11-bp direct repeat present in both the *MTCO1* and *MTND5* genes and the 3,715-bp deletion (panel B) occurs at a 10-bp direct repeat present in both the *MTRNR1* and *MTND2* genes. Additionally, the frequency of detection of these novel deletions approached that of the 4,977-bp ''common'' deletion.

A novel mtDNA species containing three deletions was identified in an 82-year-old patient (Figure 3). In this mutation, 10 or 21 bp are retained, while 1,647, 591, and 3,572 bp are deleted. Only one of the three deletions contains a direct repeat of only 3 bp.

Two deletions that contain the same 5′ breakpoint were observed in the margin samples: 3,715 bp (1,491:5,206 D10/10) and 3,833 bp (1,491:5,322 D9/9) (Table 1). The 9-bp direct repeat occurs four times in the human mtDNA at positions 1,491, 5,206, 5,323, and 10,087. However, other deletion products such as 1,491:10,087, 5,206:5,323, 5,206:10,087, or 5,323:10,087 were not observed.

Quantification of deletions

To determine the heteroplasmy levels of the common deletion in margin samples, we used real-time PCR. Some of the margin samples were chemically treated to allow for the separation of dermis from epidermis using microscopic dissection. DNA from these samples was isolated and amplified using primers flanking the breakpoints in the case of wild-type and mutant species (Materials and Methods). Figure 4 shows the quantitative analysis for the ''common'' 4,977-bp deletion. The heteroplasmy level is plotted against patient age. Levels of heteroplasmy from the dermis are shown in red, from epidermis, green, and from unsplit samples, black. Split samples from the same patient are indicated by squares and triangles. For the 4,977-bp ''common'' deletion, there is a positive correlation between patient age and heteroplasmy level when all data points are considered (Spearman rank correlation coefficient $\rho = 0.31$, $P = 0.045$). We also observed that there was a consistently higher level of heteroplasmy of the 4,977 bp ''common'' deletion in dermis as compared to epidermis from the same excised sample when split specimens were analyzed.

DISCUSSION

While numerous deletions were observed in margin samples, fewer were observed in the tumors (Figure 1). Screening for mtDNA deletions detected the 4,977-bp ''common'' deletion (Table 1), which is reported to be one of the biomarkers of photoaged skin (Birch-Machin *et al.*, 1998). In agreement with this theory, there was a positive correlation between the level of heteroplasmy of this deletion and the patient's age (Figure 4). Deviations from this linear relationship may be secondary to variations in levels of accrued sun exposure; as such information was not available for this study. We found a consistently higher level of heteroplasmy of the 4,977-bp ''common'' deletion in the dermis as compared to the epidermis in our split samples, consistent with previous studies (Birch-Machin *et al.*, 1998). In addition to the 4,977-bp ''common'' deletion, we have found a number of previously unreported deletions (Table 1), including novel 3,715- and 6,278-bp deletions present in numerous photoaged skin samples. While these deletions have not been previously reported, they are structurally similar to the 4,977-bp ''common'' deletion in that they generally contain short direct or indirect repeats at the breakpoints, and a single copy of the repeat is left behind in the deleted molecule. The structural similarities between the mtDNA deletions observed in the skin and those seen in tissues not exposed to UVR (Kogelnik *et al.*, 1996) imply a potential common factor in their generation or resolution. Furthermore, the number of novel deletions in photoaged skin not reported in other tissues suggests that skin may be more vulnerable to such mutations via direct exposure to UVR.

Table 1. MtDNA deletions detected

Age	Type	Breakpoints	Size (bp)	Fl. Rep.	Genes deleted	Ref.
44	Novel	1,491:5,206[1]	3,715	D 10/10	MTRNR1-MTND2	0
51	Reported	534:4,430	3,895	D 13/13	MTTFH-MTTM	3
	Novel	7,682:13,722	6,039	NR	MTCO2-MTND5	0
54	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
	Novel	7,468:1,5989	8,521	D 9/9	MTTS1-MTTP	0
54	Novel	1,491:5,206[1]	3,715	D 10/10	MTRNR1-MTND2	0
	Novel	1,491:5,323	3,831	D 9/9	MTRNR1-MTND2	0
62	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
	Novel	7,817:16,075	8,257	D 2/2	MTCO2-MTDL	0
	Novel	7,663:13,804	6,140	D 5/5	MTCO2-MTND5	0
62	Novel	7,398:13,677[2]	6,278	D 11/11	MTCO1-MTND5	0
65	Novel	1,491:5,206[1]	3,715	D 10/10	MTRNR1-MTND2	0
67	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
69	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
	Novel	7,957:13,370	5,412	D 4/4	MTCO2-MTND5	0
71	Novel	7,398:13,677[2]	6,278	D 11/11	MTCO1-MTND5	0
	Novel	8,131:13,570	5,438	I 12/14	MTCO2-MTND5	0
	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
73	Novel	1,491:5,206[1]	3,715	D 10/10	MTRNR1-MTND2	0
	Novel	7,398:13,677[2]	6,278	D 11/11	MTCO1-MTND5	0
	Novel	7,853:13,887	6,033	NR	MTCO2-MTND5	0
76	Novel	7,398:13,677[2]	6,278	D 11/11	MTCO1-MTND5	0
	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
78	Novel	8,299:13,153	4,853	I 8/9	MTTK-MTND5	0
80	Novel	7,398:13,677[2]	6,278	D 11/11	MTCO1-MTND5	0
81	Novel	8,137:14,771	6,633	D 3/3	MTCO2-MTNC9	0
82	Novel	7,912:9,560[3]	1,647	NR	MTCO2-MTCO3	0
	Novel	8,568:12,976	4,407	D 8/8	MTATP6-MTND5	0
	Novel	9,570:10,162[3]	591	NR	MTCO3-MTND3	0
	Novel	10,183:13,756[3]	3,572	D 3/3	MTND3-MTND5	0
82	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
	Novel	8,525:13,716	5,189	NR	MTATP8-MTND5	0
85	Common	8,469:13,447	4,977	D 13/13	MTATP8-MTND5	96
	Novel	7,728:13,806	6,077	I 13/15	MTCO2-MTND5	0
	Novel	8,030:14,334	6,303	D 3/3	MTCO2-MTND6	0
85	Novel	8,464:13,138	4,673	NR	MTATP8-MTND5	0
88	Novel	8,441:14,505	6,063	I 13/15	MTATP8-MTND6	0

Deletions were identified in 20 of 29 patient samples. The patient's age, deletion type, deletion breakpoints, deletion size, flanking repeats (Fl. Rep.), genes deleted, and number of references in the Mitomap database (Ref.) are shown. D: direct repeat. I: indirect repeat. NR: no repeat.
[1]Novel 3,715-bp deletion found in high frequency.
[2]Novel 6,278-bp deletion found in high frequency.
[3]Complex triple deletion.

There was a striking difference between the mtDNA deletion patterns observed in tumors as compared to margins. Using long-extension PCR, mtDNA deletions were readily detected in the margin skin of the patients undergoing NMSC surgery. However, we observed far fewer mtDNA deletions in the tumors compared to margins, which was a statistically significant finding. In fact, the tumors often had no detectable mtDNA deletions despite the fact that long-extension PCR

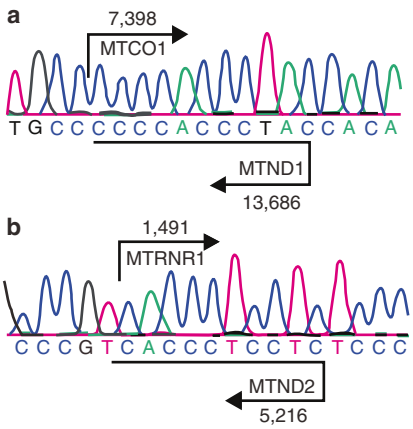

Figure 2. The sequences of the (a) 6,278-bp and (b) 3,715-bp deletion breakpoints from representative samples are shown. In the 6,278-bp deletion, there is a direct repeat of 11 bp (CCCCACCCTAC, underlined) in the *MTCO1* and *MTND5* genes; the breakpoints are 7398:13,677. In the 3,715-bp deletion, there is a direct repeat of 10 bp (CACCCTCCTC, underlined) in the *MTND2* and *MTRNR1* genes; the breakpoints are 1,489:5,206.

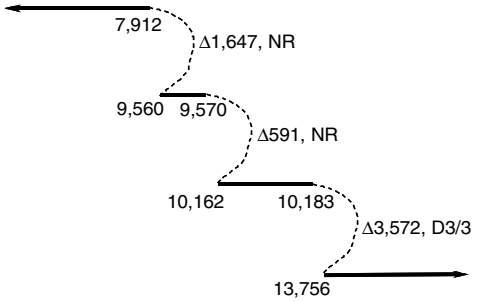

Figure 3. Complex triple deletion. A novel complex triple deletion was detected from the skin of an 82-year-old patient. The deletion breakpoints were sequenced and are shown under the solid lines. The deletions (dotted lines) are identified by "Δ" and their size. D: direct repeat at the deletion breakpoint. NR: no repeats at the deletion breakpoint.

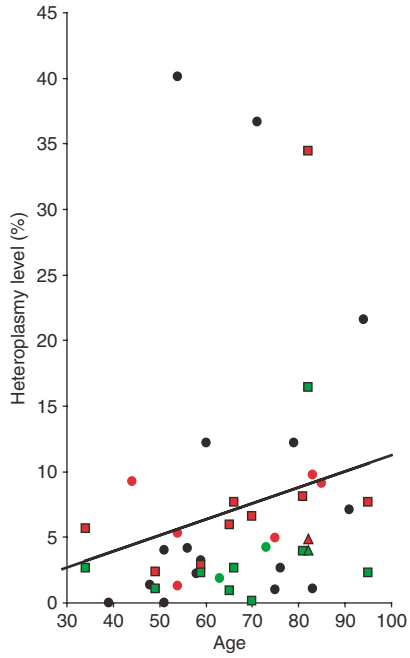

Figure 4. 4,977-bp deletion heteroplasmy levels. The percent heteroplasmy as quantified by real-time PCR is plotted against patient age. Black circles indicate unsplit samples. For split samples, red indicates dermis and green epidermis. Dermis and epidermis from the same patient are indicated by squares or triangles. A best-fit line is added (linear trendline in Microsoft Excel), the correlation coefficient is 0.21.

may be biased toward preferential amplification of smaller (deleted) products (Kajander *et al.*, 1999). Deletions that were observed in tumors were also found in the corresponding margins. This observation is likely due to the nature of the Mohs surgical excision procedure, which generated margin specimens that were histologically confirmed to be tumor-free, while the tumor samples themselves potentially contained an admixture of malignant and nonmalignant cells. Taken together, these data support the idea that there is no specific mtDNA deletion signature in NMSC tumors (Yang *et al.*, 2004). The presence of full-length mtDNA in these tumors, despite the fact that they are arising in areas of skin characterized by abundant mtDNA deletions, may be indicative of a selective process for full-length mtDNA in the process of NMSC tumorigenesis. Alternatively, the absence of mtDNA deletions in tumors may indicate a potential protective role for mtDNA deletions in keratinocytes. Perhaps the accumulation of mtDNA deletions during skin aging creates a bioenergetic profile that prohibits the damaged keratinocytes from undergoing a malignant transformation. It is also possible that high levels of mtDNA deletions in a proliferating malignant keratinocyte may trigger apoptosis. These findings also argue against theories proposing that stochastic segregation of mutant mtDNAs can lead to mutant mtDNA homoplasmy in tumors (Coller *et al.*, 2001).

The mechanism of formation of such mtDNA deletions has been proposed to be a slip mispairing of the repeats during replication (Schon *et al.*, 1989; Shoffner *et al.*, 1989; Mita *et al.*, 1990; Berneburg *et al.*, 1999; Samuels *et al.*, 2004). In order for such a mutation to occur, however, both breakpoints must be single stranded simultaneously, which does not normally occur. However, the sequences flanking the repeats or the sequences within the repeats may render the DNA susceptible to structural conformations allowing mispairing (Hou and Wei, 1996). Our findings show that most deletions identified contain such repeats (Table 1), supporting the slip replication model. The novel 3,715- and 6,278-bp deletions contain 10- and 11-bp direct repeats, respectively, similar in size to that of the 4,977-bp "common" deletion (13 bp). Additionally, the novel 6,278-bp deletion contains a homopolymeric track of seven consecutive cytosine residues interrupted by a single adenosine residue within its repeated breakpoint sequence. Such sequences have been proposed to take on structural characteristics allowing for mispairing to occur (Fullerton *et al.*, 2001). These features may explain the reason why the frequencies of detection of the novel 3,715- and 6,278-bp deletions approached that of the 4,977-bp "common" deletion. It has been proposed that ROS, which are normally generated in response to UVR

(Scharffetter-Kochanek *et al.*, 1997), play a role in the generation of the 4,977-bp ''common'' deletion (Berneburg *et al.*, 1999). Thus, it is not surprising that so many unreported mtDNA deletions were identified in photoaged skin given its role as a barrier to UVR. In addition, nucleotide excision repair may be deficient in mitochondria (Clayton *et al.*, 1974). This deficiency may result in increased mtDNA damage in the form of DNA photoadducts in tissues exposed to UVR. These photoadducts may trigger the formation of mtDNA deletions, although the specific mechanism for this possibility is unclear. It is likely that persistent mtDNA photoadducts would block mtDNA replication and may be a mechanism by which mutated mtDNAs are ultimately diluted from replicating mtDNAs in cell division (Clayton *et al.*, 1974).

An unreported complex triple deletion was identified on a single strand of mtDNA (Figure 3 and Table 1). This specimen was examined for intermediate deleted products, but none were found (data not shown). Furthermore, the breakpoints of this deletion were atypical in that two of the three deletions had no repeats and one had a 3-bp direct repeat, suggesting a different mechanism of formation. Perhaps this type of deletion is indicative of a more serious mtDNA insult than typical UV exposure, such as from a different environmental or systemic insult.

While the 4,977-bp ''common'' deletion has been proposed to be a biomarker for photoaging, it may reflect only a small portion of total mtDNA damage. For example, the frequencies of detection of the 3,715- and 6,278-bp deletions approach that of the 4,977-bp ''common'' deletion, implicating that these deletions may prove valuable as additional markers of photoaging in the skin. Additionally, tumor-free photoaged skin contains an abundance of various deletions (Table 1), not merely the novel 3,715- and 6,278-bp deletions nor even the 4,977-bp ''common'' deletion. Thus, the use of the 4,977-bp ''common'' deletion as a biomarker for photoaged skin may indeed be the tip of the iceberg.

These data are consistent with previous studies suggesting the potential use of mtDNA deletions as biomarkers of cutaneous photoaging (Birch-Machin *et al.*, 1998; Ray *et al.*, 2000; Koch *et al.*, 2001; Krishnan *et al.*, 2004; Yang *et al.*, 2004) in that deletions are common in photoaged skin. Deleted mtDNA products observed in tumors (such as those in the 80-year-old BCC patient (Figure 1a)) were also observed in the margin, suggesting that the tumor contained an admixture of tumor and tumor-free cells. This is consistent with previous studies showing that when tumor cells are microdissected and separated from tumor-free cells, deletions were not observed within experimental limits (Birch-Machin *et al.*, 1998). It is also consistent with a previous study showing the absence of mtDNA deletions in colorectal carcinomas (Penta *et al.*, 2001). A previous study that specifically examined mtDNA deletions in NMSC patients reported a different pattern of mtDNA deletions in the tumors compared to that in the perilesional skin (Durham *et al.*, 2003). In their study, Durham *et al.* (2003) examined the patterns of mtDNA deletions using an approach that divides the mtDNA into two amplifiable fragments. The total number of deleted species amplified from the perilesional tissue generally exceeded the number of deleted species identified in tumor tissue, which is consistent with our observations. Additionally, the ratio of deletions in the major arc to that of the minor arc is highly consistent between their study (20:4) and ours (20:3; Figure S1). However, Durham *et al.* (2003) often observed mtDNA deletions which appeared to be specific to the tumors, as they were not visualized in the perilesional skin. This observation is in contrast with our study that showed that mtDNA deletion patterns present in the tumor specimens were also present in the adjacent margin samples, and failed to identify mtDNA deletion species that were unique to the tumor itself. Perhaps the differences in our results and these results can be explained by the method of acquisition of surgical specimens. In the case of this study, the use of the Mohs surgical technique provides tumor tissue with minimal admixed nontumor tissue, which may explain the presence of the bands corresponding to deleted mtDNA species in the 80- and 63-year-old BCC patients (Figure 1a). It is also possible that differences in experimental design such as PCR amplification parameters may be responsible for the observed differences between these two studies.

ROS induce mtDNA deletions by causing strand breaks in the mtDNA (Shoffner *et al.*, 1989; Berneburg *et al.*, 1999). Such breaks can allow for deletions via a slip repair mechanism whereby repeated sequences are misannealed during replication and UVR-induced breaks in the DNA cause the intervening portion of the mtDNA to be deleted in the replicated copy (Schon *et al.*, 1989; Shoffner *et al.*, 1989; Mita *et al.*, 1990; Madsen *et al.*, 1993; Berneburg *et al.*, 1999). Such deletions would lead to a loss of multiple genes required for oxidative phosphorylation, usually including multiple tRNAs which are required for protein synthesis of all polypeptides encoded by the mtDNA. This deficiency of all mitochondrially encoded proteins may induce a state of respiratory deficiency at a level that does not support an adequate biochemical environment for the development of neoplasia. This may explain why mtDNA point mutations that only induce a partial respiratory deficiency in humans (Petros *et al.*, 2005) or mice (Sligh *et al.*, 2000) are seen in association with hyperproliferative cellular phenotypes such as prostate cancer or optic nerve head hamartomas. Increased cellular ROS generated as a result of dysfunctional mitochondrial electron transport may be implicated in this neoplastic process. On the contrary, studies of mtDNA deletions present in cells or in the germ line of transgenic mice have not been associated with maintenance of a neoplastic phenotype (Inoue *et al.*, 2000; Akimoto *et al.*, 2005). As mtDNA deletions accrue in photo-aged skin, they may be useful as biomarkers of the combined effects of chronological aging and UV exposure. Additionally, specific mtDNA deletions may complement other deletions and the potential level of a single mtDNA deletion may plateau with time as it adversely affects the bioenergetic properties of the cell. Measurement of a panel of deletions may be a more useful assay of photodamage than heteroplasmy levels of any single deletion alone. An analysis of the number of different deletions

detectable by long-extension PCR, for example, may be a potential indicator of photodamage. Quantitative analysis of other deletions such as those in Table 1, particularly the 3,715- and 6,278-bp deletions (whose frequencies approach that of the 4,977-bp ''common'' deletion), in addition to the 4,977-bp ''common'' deletion may be useful for this purpose. Future studies will include correlational analyses of these two frequently occurring deletions with age.

MATERIALS AND METHODS

Tissue acquisition

In all, 29 skin cancer tumor specimens and tumor-free margin skin specimens were obtained from the Mohs micrographic surgery unit at the Vanderbilt Clinic using a protocol approved by the Vanderbilt University Institutional Review Board (25 BCC and four squamous cell carcinoma). Some excised skin specimens were soaked in 0.5 M NaBr at 4°C overnight to enable the epidermis to be physically peeled from the dermis using microscopic dissection (total of five specimens, all from patients with BCC). All work adhered to the Declaration of Helsinki Principles.

DNA isolation

Approximately 25 mg of tissue was incubated for a minimum of 8 hours at 55°C rocking in 400 μl lysis solution (100 mM Tris, pH 8.0, 50 mM EDTA, 150 mM NaCl, 0.5% SDS) supplemented with 15 μl of 15 mg/ml proteinase K solution (Roche; Indianapolis, IN). Proteins were removed using ammonium acetate. DNA was precipitated using isopropanol, washed with 70% ethanol, and resuspended in sterile 10 mM Tris, 1 mM EDTA, pH 8.0. DNAs were stored at −20°C until further use.

Long-extension PCR

PCR reactions were set up with a total volume of 50 μl per reaction using the Expand Long Template PCR System (Roche; Indianapolis, IN). Each reaction included final concentrations of 1 × buffer, 2 mM each deoxynucleoside triphosphate, and 400 nM of each primer. In all, 0.5 μl template DNA and 0.7 μl enzyme were used per reaction. Primers H15473F and H14841R were used (sequences in Table S1). Long-extension PCR was performed with an initial denaturation for 2 minutes at 92°C; followed by 10 cycles of denaturation for 10 seconds at 92°C, annealing for 30 seconds at 62°C, and elongation for 18 minutes at 68°C, followed by 20 cycles of denaturation for 10 seconds at 92°C, annealing for 30 seconds at 62°C, and elongation for 18 minutes 10 seconds + 10 seconds per cycle at 68°C. PCR products were stored at 4°C until further use.

Field inversion gel electrophoresis

Long-extension PCR products were resolved on a 20-cm-long 0.75% agarose gel at 125 V with the buffer recirculating for 20–24 hours. The electric field was inverted using a PPI-200 Programmable Power Inverter (MJ Research; Watertown, MA) programmed to optimally resolve fragments from 0.5 to 40 kb using Gel Times software for Macintosh (MJ Research; Watertown, MA). The gel was initially run for approximately 15 minutes without field inversion. Products were visualized with ethidium bromide staining.

Deletion breakpoint analysis

To determine the breakpoints of the deletions identified in the long-extension PCR experiment, traditional PCR was used with distantly spaced primers and short extension times. In this way, only samples containing deletions would produce a product. PCR reactions were set up with a total volume of 25 μl per reaction. Each reaction included final concentrations of 1 × buffer, 1.5 mM $MgCl_2$, 200 nM each deoxynucleoside triphosphates, and 200 nM of each primer. Also, 0.5 μl template DNA and 2.5 U Taq polymerase (Promega; Madison, WI) were used per reaction. Primer sequences are listed in Table S1. PCR parameters were set up with an initial denaturation for 2–5 minutes at 94°C, followed by 30–35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds, and elongation at 72°C. Annealing temperatures and elongation times for the various primer pairs are listed in Table S1. A final elongation was for 5 minutes at 72°C. PCR products were stored at 4°C until further use. PCR products were resolved on agarose gels to determine their size.

DNA sequencing

PCR products were resolved on agarose gels and deleted fragments were gel purified using Gel Extraction kits (Qiagen; Santa Clarita, CA) or incubation with exonuclease I (New England Biolabs; Beverly, MA) and shrimp alkaline phosphatase (Roche; Indianapolis, IN) at 37°C for 30 minutes, followed by heat inactivation at 85°C for 15 minutes. The DNAs were sequenced using one or both of the primers used to amplify them using the ABI Prism 3700.

Real-time PCR

Real-time PCR was used to detect the heteroplasmy level for the 4,977-bp deletion. Real-time PCR reactions were set up using the iQ SYBR Green system (Bio-Rad; Hercules, CA) with a total volume of 20 μl per reaction. Each reaction included final concentrations of 1 × iQ SYBR Green Supermix (Bio-Rad; Hercules, CA) and 500 nM of each primer. Equal amounts of template DNA was used for wild type and deletion. Primers used were H8388F, H8493R, and H13477R. The sequences are: H8388F, 5′-ATGGCCCACCATAATTACCC-3′; H8493R, 5′-TTTTATGGGCTTTGGTGAGG-3′; and H13477R, 5′-GCTAATGCTAGGCTGCCAAT-3′. These primers were designed to span one breakpoint of the 4,977-bp deletion. To determine the quantity of wild-type mtDNA, the primers H8388F and H8493R were used. To determine the quantity of mtDNA containing the 4,977-bp deletion, the primers H8388F and H13477R were used. Since the elongation time is short (30 seconds), PCR products corresponding to wild-type mtDNA were not amplified when H8388F and H13477R were used (data not shown). The heteroplasmy level was calculated as the quantity of deleted mtDNA divided by the sum of the wild-type mtDNA and the deleted mtDNA. Real-time PCR was performed on an iCycler (Bio-Rad; Hercules, CA) with an initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing 30 seconds at 55°C, and elongation for 30 seconds at 72°C, followed by a melt curve analysis from 95°C to 4°C in 0.5°C increments for 10 seconds per step. PCR products were resolved on agarose gels to ensure that the products were of appropriate size.

Statistical methods

Continuous variables that were normally distributed are described by the mean and SD, while those not normally distributed are summarized by the median and interquartile range. The Shapiro–Wilk test was used to determine if continuous variables were

normally distributed. The Wilcoxon signed-rank test was used to compare non-normally distributed continuous variables in paired samples (number of tumor deletions versus number of margin deletions in the same individual). Spearman rank correlation was used to measure the strength of association between two non-normally distributed continuous variables. The level of significance was set at 0.05. All statistical analyses were performed using STATA 8.0 (College Station, TX).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Characterized deletions. The deletions characterized in Table 1 are presented as horizontal bars. Genes are shown above. *Complex triple deletion (Figure 3).

Table S1. Primers, annealing temperatures, and elongation times used to amplify mtDNAs identified to contain deletions. Primers are listed in pairs in which they were used. AT: Annealing temperature, given in °C. ET: elongation time, given in seconds.

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