Involvement of mtDNA Damage Elicited by Oxidative Stress in the Arsenical Skin Cancers

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Arsenic causes several human cancers. Arsenic-induced Bowen’s disease (As-BD), the most common arsenical cancer, is characterized by increased proliferation, dysplasia, and individual cell apoptosis, all of which involve mitochondria. We reported that arsenic causes aberrant keratinocyte proliferation through mtTFA-mediated mitochondrial biogenesis in As-BD. Increasing mitochondrial biogenesis causes cells to undergo oxidative stress. However, how arsenic induces oxidative stress and causes mtDNA damage in arsenical cancers remains largely unknown. Using tissues from As-BD patients and arsenic-treated keratinocytes, we determined the oxidative stress, antioxidant enzymes, DNA-repair enzymes, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) level in mtDNA by immunofluorescence, real-time PCR, and western blot. The results showed that oxidative stress was enhanced in both As-BD and arsenic-treated keratinocytes. Antioxidant enzymes including manganese-superoxide anion and copper/zinc-superoxide anion and DNA-repair enzymes were upregulated concomitantly in tissues and cells. In arsenic-treated keratinocytes, increased mitochondrial oxidative stress and the 8-OHdG level in mtDNA were attenuated by pretreatment with ascorbic acid, a potent antioxidant. Further, we found several somatic mutations in the ND4, NDS, and ND6 genes of mtDNA in lesional but not in perilesional skin from As-BD patients. Taken together, the results suggest that oxidative damage and mutations to mtDNA might be involved in the arsenical skin cancers in the context of mitochondrial biogenesis.

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INTRODUCTION

Arsenic is the 20th most common element in the earth’s crust. Arsenic has been designated as a Class I carcinogen by (IARC 2004). Long-term exposure to arsenic can cause human cancers in the skin, lung, bladder, and liver. Among these cancers, skin cancers are the most common (Yeh, 1973). The prevalence odds ratio to develop skin cancers increases proportionally to the concentrations of arsenic in well water in West Bengal (Haque et al., 2003), Bangladesh (Ahsan et al., 2006), and Inner Mongolia (Guo et al., 2006). The arsenical skin cancers include Bowen’s disease (carcinoma in situ), squamous cell carcinoma, basal cell carcinoma, and mixed forms (Yeh et al., 1968; Abernathy et al., 1999; Centeno et al., 2002). Among these skin cancers, Bowen’s disease is the most common. Arsenic-induced Bowen’s disease (As-BD) tends to affect multiple sites predominantly distributed in sun-spared skin, whereas non-As-BD disease (or UV-induced) tends to affect a solitary site in the sun-exposed skin (Lee et al., 2004). In addition, in patients with As-BD, there are usually variegated hyperpigmations on the back and chest with characteristic palmar plantar arsenic keratosis (Tseng et al., 1968). In contrast, the classical Bowen’s disease is mostly located in sun-exposed skin without such pigimentary abnormalities and palmar plantar keratosis (Yeh et al., 1968). Microscopically, As-BD is characterized by increased proliferation, dysplasia, and occasional apoptosis. Although similar in histopathology, As-BD and classical UV-induced Bowen’s disease have distinct pathophysiological mechanisms in apoptosis of affected cells (Lee et al., 2004), immune interactions (Liao et al., 2009), and mitochondrial biogenesis (Lee et al., 2011; Martinez-Outschoorn et al., 2011). The carcinogenic mechanism of arsenical skin cancers has not been fully understood. Arsenic acts as a double-edged sword in the progression of cancers. At one end, it is the treatment of choice for acute promyelocytic leukemia (Kamimura et al., 2011), and at the other end, it causes several kinds of cancers. Interestingly, at lower doses, arsenic promotes cell proliferation, but at higher doses, arsenic induces cell death in various cell types (Yu et al., 1992; Gupta et al., 2003; Liao et al., 2013).
et al., 2004). Mitochondria appear to be a reasonable target in the process of arsenical carcinogenesis because cell proliferation (Martinez-Outschoorn et al., 2011), cell death (Lee and Wei, 2000), and abnormal cell differentiation (Chen et al., 2010a) all require the participation of mitochondria. In fact, genetic and metabolic alterations in mitochondria have been shown to be the cause or contributory factors of carcinogenesis (Modica-Napolitano and Singh, 2002; Petros et al., 2005; Ishikawa et al., 2008). Many cancers are characterized by mitochondrial abnormalities that are associated with disease progression and patient survival (Petros et al., 2005; Ishikawa et al., 2008). Mutations and damage to mtDNA were found in solid tumors as well as in lymphoma-leukemia (Czarnecka et al., 2010). Oxidative stress-induced mutation and damage of mtDNA, and mitochondrial dysfunction are thought to have an important role in carcinogenesis. This notion was supported by the findings that mutated cancer-causing proteomic alteration feedback amplified the cell transformation process by directly affecting mitochondrial function and promoted a vicious spiral of malignant cell transformation (Kulawiec et al., 2006).

The peripheral blood mononuclear cells of patients with arsenical skin cancers had elevated levels of intracellular reactive oxygen species (ROS), mitochondrial membrane permeability, and increased cytochrome-c release, leading to activation of caspase cascades and apoptosis (Banerjee et al., 2008). We have previously demonstrated that low doses of arsenic-induced mtTFA-mediated mitochondrial biogenesis, resulting in aberrant cell proliferation in arsenical skin cancers and in arsenic-treated keratinocytes (Lee et al., 2011). This is supported by a recent study demonstrating that chronic exposure to arsenic increases cell survival in human prostate epithelial cells through mtTFA (Singh et al., 2011). Therefore, we have contended that upregulation of biogenesis and function of mitochondria are involved in arsenic carcinogenesis. However, increased mitochondrial biogenesis and respiratory function might also increase the chance to develop oxidative stress that can render the mtDNA more susceptible to oxidative damage and mutation. Consequently, the sustained mtDNA damage would interfere with the mitochondrial function leading to the overproduction of ROS in the mitochondria of arsenic-exposed cells. Partridge et al. (2007) reported that arsenic can reduce the mitochondrial biogenesis, cytochrome-c oxidase activity, and oxygen consumption in Chinese hamster ovary-derived cells. However, the direct target cells in arsenic carcinogenesis in the skin are keratinocytes that originate from ectoderm, but not endoderm, that develops into ovary in embryogenesis. Further, the Chinese hamster ovary-derived cells are distinct from primary keratinocytes because they belong to a cell line and lack EGFR expression that is essential for epidermal differentiation (Ahsan et al., 2009). The effects of arsenic on cells appear to be dependent on cell type. We have reported that low doses of arsenic induce mitochondrial biogenesis, increase oxygen consumption, and induce cytochrome-c oxidase function in both As-BD disease and arsenic-treated primary keratinocytes (Lee et al., 2011). Thus, the role of oxidative stress and mtDNA damage in the pathogenesis of arsenical cancers in primary keratinocytes has never been well addressed. Therefore, this study was aimed to answer whether arsenic could induce mitochondrial oxidative stress, leading to oxidative damage and instability of mtDNA in arsenical cancers in the context of increased mitochondrial biogenesis. We also examined whether the ROS scavengers would be altered in response to arsenic-induced mitochondrial oxidative stress and subsequent mitochondrial damage.

RESULTS
Increased oxidative stress and oxidative DNA damage in arsenical skin cancers
To study whether oxidative damage is present in arsenical cancers, we measured the contents of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in DNA of skin tissues from As-BD, non-As-BD patients, and age-matched normal controls by immunofluorescence staining. The results showed that the 8-OHdG level in skin DNA was increased in As-BD patients as compared with those in normal subjects and non-As-BD patients, respectively (Figure 1a). We also investigated whether the expression levels of antioxidant enzymes were elevated to accommodate the increased oxidative stress in As-BD. We determined and compared the expression levels of copper/zinc-superoxide anion (Cu/Zn-SOD), manganese-superoxide anion (Mn-SOD), and catalase in skin tissues of As-BD patients, non-As-BD patients, and normal subjects by immunofluorescence. The results showed that the expression levels of Cu/Zn-SOD and Mn-SOD were increased (Figure 1b and c), whereas the expression of catalase was slightly reduced in skin tissues from As-BD patients compared with those from non-As-BD patients or normal subjects (Figure 1d). These findings indicate that the oxidative stress level was increased, accompanied with an imbalanced expression of antioxidant enzymes, which may account for the increased oxidative DNA damage in As-BD.

Increased expression of antioxidant enzymes and DNA-repair enzymes in arsenical skin cancers
We then investigated whether the above finding of the altered expression of antioxidant enzymes could be observed at the transcriptional level in skin tissues of As-BD patients. The expression levels of DNA repair-related enzymes in skin tissues of As-BD patients were also investigated. To achieve this goal, we profiled the expression levels of antioxidant enzymes and DNA-repair enzymes by real-time quantitative PCR (RT-QPCR) in lesional and perilesional skin tissues from As-BD patients. Among the six antioxidant enzymes or proteins examined (Cu/Zn-SOD, Mn-SOD, catalase, glutathione peroxidase-1, thioredoxin-1, and peroxiredoxin-1), except a slight reduction in catalase, all of the other five antioxidant enzymes were upregulated in lesional skin tissues compared with those in perilesional skin tissues of As-BD patients (Figure 2a). Moreover, all of the five DNA-repair enzymes (hOGG1, p53, XPC, ERCC1, and GADD45) were significantly upregulated in lesional skin tissues compared with those in perilesional skin tissues of As-BD patients (Figure 2b). These findings indicate that the
expression levels of antioxidant enzymes and DNA-repair enzymes were altered in the cancerous tissues but not in the perilesional skin from the As-BD patients, suggesting a cellular defense against oxidative stress in the arsenical skin cancers.

Alterations in the expression of antioxidant enzymes and DNA-repair enzymes in arsenic-treated human keratinocytes

To investigate whether the oxidative stress, oxidative damage, altered expression of antioxidant enzymes, and DNA-repair enzymes observed in skin tissues of As-BD patients could be reproduced in vitro, we treated human keratinocytes with arsenic (0, 0.5, 1, or 5 μM) for 24 hours and measured the expressions of the above antioxidant enzymes and DNA-repair enzymes by RT-QPCR. There was a dose-dependent increase in the mRNA levels of both families of enzymes in arsenic-treated keratinocytes (Figure 3a and b). The expression level of Mn-SOD was most significantly increased among the antioxidant enzymes in skin tissues of As-BD patients and increased in arsenic-treated keratinocytes in a dose-dependent manner (Figure 3a). However, the expression of catalase was dramatically decreased in keratinocytes treated with a high concentration (5 μM) of arsenic. The results from arsenic-treated keratinocyte were consistent with those observed ex vivo, which revealed a cellular defense against oxidative stress. Furthermore, we measured the protein expression of antioxidant enzymes in arsenic-treated keratinocytes by western blot. The results revealed a dose-dependent increase of the expression of antioxidant enzymes in
expression of antioxidant enzymes could be reproduced in vitro, and keratinocytes can be used in the subsequent mechanistic studies.

Enhanced mtDNA damage by oxidative stress in skin of As-BD patients and in arsenic-treated keratinocytes

We reported that mitochondrial biogenesis was enhanced in arsenical skin cancers and in arsenic-treated keratinocytes (Lee et al., 2011). Because we found a general increase in oxidative stress and inductions of the antioxidant enzymes, we then investigated whether mitochondria, a major source of oxidative stress in human cells, are vulnerable to oxidative damage while undergoing substantial mitochondrial biogenesis. To address this, we measured the oxidative damage to mtDNA in As-BD and in arsenic-treated keratinocytes, respectively. The results revealed that the level of oxidative damage to mtDNA was significantly increased in lesional skin as compared with the perilesional skin from As-BD patients (Figure 4a). Furthermore, there was a dose-dependent increase of mtDNA damage in arsenic-treated keratinocytes (Figure 4b). The induction of mtDNA damage in vitro could be abolished when keratinocytes had been treated for 4 hours with 100 μM ascorbic acid, a potent antioxidant (Figure 4b). These findings indicate that mitochondrial oxidative stress may mediate oxidative damage to mtDNA in arsenical skin cancers.

Somatic mutations of mitochondrial genome in the lesional skin from As-BD patients

We found increased oxidative mtDNA damages in the lesional skin from As-BD patients. We then asked whether there were mtDNA mutations consistent with oxidative damage in tissues of patients with As-BD. The entire mitochondrial genome was directly sequenced to identify the somatic mutations in the lesional As-BD skin as compared with that in perilesional skin tissues to further contend that mtDNA mutation-elicited oxidative stress involved in the carcinogenesis of arsenical skin cancers. The result showed that five somatic mutations in the mitochondrial genome occurred in for out of six As-BD patients (Figure 2 and Supplementary Figure 1 online). Table 1 summarizes these somatic mutations of mtDNA were identified from As-BD patients. Of these, A14230G, T11076A, C12405, and T14783C are homoplasmic mutations, and A11035T is a heteroplasmic mutation of mtDNA. The A14230G, T11076A, C12405, and T14783C are homoplasmic mutations, identified from As-BD patients. Of these, A14230G, T11076A, C12405, and T14783C mutations are located in the ND4, ND5, and ND6 genes that encode the key components of mitochondrial Complex I. The T14783C mutation is on the Cyt b gene, one of the essential components of mitochondrial Complex III. Except for C12405 and T14783C mutations being synonymous, other mutations can cause a substitution of amino acid (A14230G, A11035T) or premature termination (T11076A). The result showed that the somatic mutations of mtDNA are present in two-thirds of the As-BD lesion. These mutations can occur in the genes encoding the major component of mitochondrial respiratory enzyme Complex I and they are different from the large-scale deletions of mtDNA that are commonly induced by UV irradiation (Krishnan et al., 2004; Eshaghian et al., 2006).

![Graph](image_url)
Mitochondria-elicited oxidative stress may enhance mtDNA damage in arsenical skin cancers

Because we have found an increase of oxidative stress, oxidative mtDNA damage, and mtDNA mutations in the skin tissues of As-BD patients and in arsenic-treated keratinocytes, we then examined whether oxidative stress is increased in mitochondria of the affected cells. If so, would antioxidants attenuate the mitochondrial oxidative stress that contributed to mtDNA damage? To address this, we treated keratinocytes with arsenic at 0, 0.1, and 1 μM for 72 hours, and measured the oxidative stress by staining the cells with MitoSOX, a sensitive indicator for mitochondrial ROS. We then pretreated keratinocytes with ascorbic acid, a potent ROS scavenger for 4 hours before treatment of keratinocytes with arsenic for 72 hours. The results showed that the fluorescence of MitoSOX was apparent in keratinocytes treated with either 0.1 or 1 μM of arsenic (Figure 5a–c). The increase in mitochondrial oxidative stress by arsenic could be blocked when keratinocytes had been pretreated with 100 μM ascorbic acid for 4 hours (Figures 5d–f), suggesting that arsenic induced the increase of mitochondrial oxidative stress as a result of imbalance between oxidative and reducing events.

DISCUSSION

In this study, we demonstrated that the arsenic-elicited mitochondrial oxidative stress with compensatory antioxidant responses is accompanied by the accumulation of oxidative damage and mutation of mtDNA in arsenical cancers. Mitochondria seem to be a reasonable target in the process of arsenical carcinogenesis because pathological features of As-BD, including cell proliferation, cell death, and dysplasia all require the participation of mitochondria. Our findings support the notion that oxidative stress-elicited mtDNA damage and mutation contribute to arsenical carcinogenesis and treatment of keratinocytes with antioxidants such as ascorbic acid could ameliorate the arsenic-induced mitochondrial oxidative stress and oxidative damage to mtDNA (Figure 5g).

Oxidative damage and instability of mtDNA occur concurrently with increased ROS in several cancers (Ralph et al., 2010). The majority of previous studies investigated the role that ROS has in carcinogenesis with regard to their pro-apoptotic effects. However, how ROS get involved in oxidative damage and mutation of mtDNA in arsenic carcinogenesis remains unknown. ROS are able to induce...
oxidative damage to DNA, RNA, and proteins, and thereby render cells susceptible to mutagenesis and carcinogenesis. The mitogenic potential of ROS was revealed by the observation that ROS stimulated cell proliferation of hepatoma via the cross-talk between PI3K and JNK pathways, which in turn enhance protein expression of c-Fos and c-Jun (Liu et al., 2002). Meanwhile, mitochondria are the main source of ROS in human cells (Berneburg et al., 2006). It was reported that oxidative damage to nuclear DNA were increased in HeLa cells with defective mitochondria and mtDNA-depleted ρ<sup>0</sup> cells (Delsite et al., 2003). A study using Caenorhabditis elegans showed that mitochondrial oxidative stress results in nuclear hypermutability (Hartman et al., 2004). Furthermore, mitochondrial genome instability and mitochondrial ROS could enhance the intestinal tumorigenesis in a mouse adenomatous polypl model (Woo et al., 2012). Based on this study, we contend that the accumulation of oxidative damage in mtDNA by arsenic-elicited mitochondrial ROS production may lead to a "vicious cycle", which in turn affects the instability of the mitochondrial genome and causes the mutation of mtDNA, subsequently generating more mitochondrial ROS through impaired respiratory function of mitochondria (Figure 5g). Our results suggest that arsenic increases mitochondrial oxidative stress, potentiating mtDNA damage and mutation, which might drive the carcinogenic progression in arsenical cancers.

Arsenic causes oxidative stress in several types of cancer cells, mostly at concentrations higher than 5 μM. The majority of arsenic-induced ROS is generated from mitochondria, as revealed by the observation that Complex I inhibitor completely abrogated the increase of ROS induced by arsenite in murine keratinocytes (Corsini et al., 1999). We showed previously that mitochondrial biogenesis is critical in promoting aberrant proliferation of arsenic-treated human primary keratinocytes (Lee et al., 2011). The expression of factors regulating mitochondrial biogenesis, including PGC-1α, NRF-1, and mtTFA, are increased in arsenic-treated human primary keratinocytes. It was reported that the upregulation of PGC-1α not only increases mitochondrial biogenesis but also enhances the antioxidant defense system in vascular endothelial cells to coping with oxidative stress (Valle et al., 2005). We showed that arsenic-induced stress response in affected cells includes not only the increase of mitochondrial biogenesis but also an upregulation of antioxidant enzymes and DNA-repair enzymes in primary keratinocytes and in arsenical skin cancers. Most importantly, in the lesional skin tissues of patients with arsenical cancers, we found five point mutations of mtDNA, which are located on the ND4, ND5, ND6, and Cyt b genes. Because these somatic mutations are located on the coding region of Table 1. Summary of somatic mtDNA mutation in the paired lesional and perilesional skin tissues from As-BD patients

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Mutation</th>
<th>Gene</th>
<th>Amino-acid change</th>
</tr>
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<tbody>
<tr>
<td>BD1</td>
<td>A→G</td>
<td>ND6</td>
<td>Tyr (ATA)→Val (GTA)</td>
</tr>
<tr>
<td>BD2</td>
<td>A→G</td>
<td>ND6</td>
<td>Tyr (ATA)→Val (GTA)</td>
</tr>
<tr>
<td>BD4</td>
<td>T→A</td>
<td>ND4</td>
<td>Leu (TAA)→stop codon (TAA)</td>
</tr>
<tr>
<td></td>
<td>A→T</td>
<td>ND4</td>
<td>Lys (AAA)→Asn (AAT)</td>
</tr>
<tr>
<td>BD6</td>
<td>C→T</td>
<td>ND5</td>
<td>Leu (CTT)→Leu (CTT)</td>
</tr>
<tr>
<td>14783</td>
<td>T→C</td>
<td>Cyt b</td>
<td>Leu (TAA)→Leu (CTA)</td>
</tr>
</tbody>
</table>

Abbreviations: Asn, asparagine; BD1-BD4, four patients with Bowen’s disease; Leu, leucine; n.p., nucleotide position; Tyr, tyrosine; Val, valine.
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**a** Control  
**b** 0.1 μM As  
**c** 1 μM As  
**d** 1 mM Vit C  
**e** 0.1 μM As + 1 mM Vit C  
**f** 1 μM As + 1 mM Vit C  

**g**

- **As**
  - Keratinocyte cell
  - Cell membrane
  - Mitochondria

- **Nucleus**
  - i. Mitochondrial biogenesis-related proteins
  - ii. DNA-repair enzymes
  - iii. Antioxidant enzymes

- **mtTFA**

- **hOGG1**

- **Mn-SOD**

- **Impaired OXPHOS**
  - **Mn-SOD**
  - **O2e–**
  - **H2O2**

- **Mutagenesis**

- **ROS**

- **Arsenical skin cancer**

- **Oxidative stress**

- **Ascorbic acid (Vit C)**
mtDNA, an error-prone translation and transcription would occur and cause impairment in OXPHOS in arsenical skin cancers. Keratinocytes, the targets of arsenic carcinogenesis, are vulnerable to oxidative stress that is tightly regulated by the 
respiratory function and integrity of mitochondria. In fact, oxidative damage to mtDNA can mediate genotoxicity of arsenic in CHO cells as revealed by cybrid studies (Liu et al., 2005). Arsenic can induce mitochondrial dysfunction through generation of reactive oxygen and nitrogen species, including superoxide anions and nitrogen oxide (Beckman and Ames, 1996; Flora, 2011).

In this study, we showed an increase of antioxidant enzymes in arsenic-treated keratinocytes in a dose-dependent manner. The increase of antioxidant enzymes seems to be an adaptive response to increased intracellular and mitochondrial oxidative stresses. Although the in vitro experiments took place in a short period of time, the observed increase of ROS and antioxidant enzymes in arsenical cancers, but not in non-arsenical cancers, supports the notion that ROS has, at least in part, a role in the initiation and progression of arsenic carcinogenesis. A microarray analysis from arsenic-treated keratinocytes also demonstrated that many genes involved in antioxidant responses, including glutathione-related proteins (GCLC, GCLM, and SLC7A11), thioredoxin (TXNRD1), and the NADPH-dependent protein (PGD), were upregulated (Bailey et al., 2010). Recent studies also showed that selective knockdown of antioxidant enzymes significantly sensitized keratinocytes to arsenic cytotoxicity (Zhao et al., 2012).

We noted a disparity in the expression of antioxidant enzymes, resulting from an increased expression of Mn-SOD and Cu/Zn-SOD, but a decreased expression of catalase, in arsenical cancers (Figures 2 and 3). This disparity is not uncommon and is consistent with the study performed in mouse epithelial cells, which showed that Cu/Zn-SOD-stable transfecants were sensitive to oxidative chromosomal aberrations and DNA damage, whereas catalase-stable transfecants corrected or overcorrected the hypersensitivity of the SOD clones depending on the ratio between the activities of catalase and SOD (Cerutti et al., 1994).

Although the number of the samples assayed ex vivo was limited in this study, we obtained a consistent and reproducible trend by using assays to assess the biological effects of arsenic on the tissues and cultured cells of patients with arsenical cancers. The other limitation is the lack of long-term treatment of keratinocytes by even lower concentrations of arsenic (for example, for several weeks). An animal study might be useful to further address the long-term effects of arsenic in vivo. However, using animal models to evaluate health effects of arsenic on the human requires careful interpretation, because a number of differences in arsenic metabolism and toxicodynamics between the human and animals have been described (Cohen et al., 2006). Nevertheless, our current findings provide important information about the involvement of mitochondrial oxidative stress, oxidative damage, and mutation of mtDNA in arsenical skin cancers. Treatment of patients with antioxidants to reduce mitochondrial ROS would be a good therapeutic approach to block the progression of arsenical cancers.

We conclude that arsenic increases mitochondrial oxidative stress, which contributes to increased oxidative damage and mutations to mtDNA in keratinocytes and in tumor tissues of patients with arsenical skin cancers. These results suggest that arsenic may cause a “vicious cycle” of mitochondrial oxidative stress triggered by increased damage and mutation of mtDNA. Together with enhanced mitochondrial biogenesis in As-BD, the above-mentioned findings led us to conjecture that oxidative damage to mitochondria may drive the carcinogenic progression in arsenical cancers. This study also highlights the possible targets to alleviate mitochondrial oxidative stress and mtDNA damage in arsenical cancers.

**MATERIALS AND METHODS**

**Selection of patients**

A total of six patients with Bowen’s disease (As-BD patients, aged 73.2 ± 8.4 years) from endemic areas were recruited from southwestern Taiwan, where artesian water was contaminated by high concentrations of arsenic. All of them had characteristic variegated pigmentations, palmoplantar keratosis, and multiple keratotic lesions. The specimens for the control group were obtained from the non-sun-exposed skin of six age-comparable normal subjects (aged 68.3 ± 10.2 years), who did not live in the endemic areas and did not have a previous history of cancers. Another control group came from patients with Bowen’s disease without a history of arsenic poisoning (non-As-BD patients, with an age of 65.3 ± 12.1 years (n = 6)). Skin tissues were collected for immunohistochemistry and extraction of RNA and DNA. Written informed consent was obtained from all participants. The study protocol was approved by the Institutional Review Board of the Kaohsiung Medical University Hospital and was adhered to the Declaration of Helsinki.

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**Figure 5. Ascorbic acid blocked the mitochondrial oxidative stress induced by arsenic in human keratinocytes.** Human keratinocytes were treated for 24 hours with arsenic at 0, 0.1, and 1 μM with (a–c) or without (d–f) 100 μM ascorbic acid (Vit C) pretreatment for 4 hours, respectively. Mitochondrial distribution and the level of mitochondrial oxidative stress were measured by staining with MitoTracker (green) and MitoSox (red), respectively. Cell nuclei were counterstained by 4’,6-diamidino-2-phenylindole (DAPI) (blue). Three repeated experiments with consistent results were obtained and representative results are shown. (g) A proposed scheme showing how arsenic-induced mitochondrial reactive oxygen species (ROS) and oxidative damage and mutation of mtDNA contribute to arsenic carcinogenesis. Besides enhancing mitochondrial biogenesis by the increased mtTFA expression (i), arsenic interferes with the mitochondrial functions by inducing oxidative damage and mutation of mtDNA through increased ROS production from the impaired mitochondrial respiration. Meanwhile, the cellular defense system, including antioxidant enzymes (ii) and DNA-repair enzymes (iii) such as manganese-superoxide anion (Mn-SOD) and human 8-oxoguanine DNA glycosylase (hOGG1), respectively, are upregulated to cope with the arsenic-induced oxidative stress in mitochondria. Of note, antioxidants such as ascorbic acid (vitamin C) protect keratinocytes from arsenic-induced mitochondrial oxidative stress and oxidative damage to mtDNA.
Chemicals and antibodies

The monoclonal antibodies against Mn-SOD, Cu/Zn-SOD, catalase, and 8-OHdG, respectively, were acquired from Abcam (Cambridge, MA) for immunohistochemical staining. The antibodies against Mn-SOD and Cu/Zn-SOD were purchased from Stressgen (Ann Arbor, MI). The antibodies against glutathione peroxidase-1, glutathione reductase, thioredoxin-1, and peroxiredoxin-1, respectively, were purchased from AbFrontier (Seoul, Korea). The mAb against β-actin was purchased from Millipore (Billerica, MA). Arsenic trioxide (As$_2$O$_3$) and ascorbic acid (vitamin C) were obtained from Sigma-Aldrich Chemical (St. Louis, MO). The fluorescent dyes of MitoSOX, Mitotracker, and 4′,6-diamidino-2-phenylindole were acquired from Molecular Probes (Eugene, OR).

Immunohistochemical staining

Sections (5 μm) of formalin-fixed, paraffin-embedded tissues were subjected to immunofluorescence staining according to our previous protocol (Lee et al., 2011). Sections were stained with primary antibodies against Mn-SOD and Cu/Zn-SOD, catalase, and 8-OHdG (1:500). The stained specimens were mounted and observed under a confocal laser scanning microscope (LSM Fluoview 500; Olympus, Shinjuku, Tokyo, Japan). Image analysis was performed by NIH image with a native plugin of “region of interest manager”. The intensity value in each region of interest was generated by pseudocolor with gray-scale transformation (0–255). Mean fluorescence intensity index of MitoSOX, Mitotracker, and 4′,6-diamidino-2-phenylindole was averaged from five random high-power fields (or region of interest).

Keratinocyte primary culture

Normal human keratinocytes were obtained from adult foreskins through routine circumcision. The keratinocytes were cultured as described previously (Lee et al., 2004). Keratinocytes at the third passage were then grown in a keratinocyte serum-free medium without BPE and rhEGF for 24 hours before experiments.

Analysis of gene expression of antioxidant enzymes and DNA-repair enzymes

The level of gene expression was determined by TaqMan-based RT-QPCR as described previously (Lee et al., 2011). Briefly, total RNA from skin biopsies and cell lysates were extracted with chloroform after adding the TRIzol reagent (Sigma-Aldrich Chemical), and then precipitated with isopropanol followed by dissolution of the RNA in diethylpyrocarbonate-H$_2$O. An aliquot of 5 μg RNA was reverse transcribed to cDNA with a Ready-to-Go RT-PCR kit (Amersham Biosciences, Uppsala, Sweden) at 42°C for at least 16 hours. RT-QPCR was performed using the LightCycler TaqMan probe-based Master kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s instructions. The primer pairs and the probes used in this study were designed by Universal ProbeLibrary Assay Design Center (Roche Applied Science) and are listed in Table 2. The expression levels of the genes of interest were normalized with the expression level of β-actin gene.

Protein extraction and western blotting

Proteins were extracted from cultured keratinocytes, and western blotting was conducted as described previously (Lee et al., 2006). Antibodies against target proteins were used at a dilution of 1:500. Specific proteins in the nitrocellulose membranes were visualized using a chemiluminescence subtraction kit (Pierce Chemicals, Rockford, IL).

### Table 2. Oligonucleotide sequences of primers and TaqMan probes used in this study

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<th>Gene name</th>
<th>Primer sequence</th>
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<tr>
<td>mtf3312</td>
<td>FW: 5′-ACCATTTCAGACGCCCCATA-3′</td>
<td>1</td>
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<td>mtr3319</td>
<td>RE: 5′-TGAATGTTGAGGCTA CGG-3′</td>
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<td>β-actin</td>
<td>FW: 5′-ATGCGCAATGAGGCCTTTC-3′</td>
<td>11</td>
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<td></td>
<td>RE: 5′-GGATGCCCAAGGAGCCCTC-3′</td>
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<tr>
<td>Mn-SOD</td>
<td>FW: 5′-TCCACTGCAAGAGAAACACG-3′</td>
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<td>RE: 5′-CTTGTTGCTTTTGAGCAGA-3′</td>
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<tr>
<td>Catalase</td>
<td>FW: 5′-CTCCCAAGAACAAGCCCTCT-3′</td>
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<td>RE: 5′-GAAGGCTGTTGTTCCGGAG-3′</td>
<td>2</td>
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<td>GPx-1</td>
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<td>RE: 5′-AGGGAATTCAGAATCTCTTCGTT-3′</td>
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<td>RE: 5′-GATCGGAAATAAGGGCGGTTG-3′</td>
<td>2</td>
</tr>
<tr>
<td>GADD45</td>
<td>FW: 5′-TGGCTTCTACTAATGACGTATTTG-3′</td>
<td>12</td>
</tr>
<tr>
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<td>RE: 5′-GCCAGGAACTTCATGTCTGTTG-3′</td>
<td>3</td>
</tr>
<tr>
<td>XPC</td>
<td>FW: 5′-GCAAGAGAAAGAAGATGAAGATAACACGG-3′</td>
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</tr>
<tr>
<td></td>
<td>RE: 5′-TGGACCCCTTTATGAAAGCT-3′</td>
<td>1</td>
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</table>

**Abbreviations:** Cu/Zn-SOD, copper/zinc-superoxide anion; ERCC1, excision repair cross complementation 1; GADD45, growth arrest and DNA-damage-inducible protein 45; GPx-1, glutathione peroxidase-1; hOOG1, human 8-oxoguanine DNA N-glycosylase 1; Mn-SOD, manganese-superoxide anion; Prx, peroxiredoxin-1; p53, tumor protein 53; Trx-1, thioredoxin-1; XPC, xeroderma pigmentosum complementation group C.

Quantitative determination of mtDNA damage

8-OHdG is one of the major ROS-induced base-modified DNA products, which has been widely accepted as a sensitive marker of oxidative DNA damage. The content of 8-OHdG in mtDNA was determined by a RT-QPCR-based technique as described previously (Chen et al., 2010b). The degree of oxidative damage to mtDNA is reflected by the abundance of 8-OHdG in mtDNA. If 8-OHdG is present at a specific region of the DNA, digestion of the 8-OHdG with using a chemiluminescence subtraction kit (Pierce Chemicals, Rockford, IL).
human 8-oxoguanine DNA glycosylase (hOGG1; R&D Systems, Minneapolis, MN) can break the DNA template at the lesion site to form an abasic site, and thus dramatically reduce the amplification of this region using PCR. The hOGG1 treatment of DNA samples was done as previously described (Eshghian et al., 2006). A volume of 1 μl of each DNA was treated with or without 1 U of hOGG1 at 37 °C for 1 hour, and the digested DNA was amplified by PCR using mtF3212 and mtR3319 primers (listed in Table 2). The PCR condition was set as follows: hot start at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 20 seconds, 62 °C for 20 seconds, and 72 °C for 20 seconds. The fluorescence intensity was measured at the end of every extension phase at 79 °C. The degree of oxidative mtDNA damage is defined as ΔCt, which is the difference between Ct-T (Ct value from DNA sample treated with hOGG1) and Ct-N (Ct value from DNA sample without hOGG1 treatment). The larger the ΔCt is, the more 8-OHdG the sample contains.

Analysis of the somatic mutations in mtDNA

mtDNA sequence was determined by direct sequencing of the PCR products amplified with the specific 11 pairs of primers to cover the entire mitochondrial genome as previously described (Tseng et al., 2011). PCR was performed for 36 cycles in 40 μl of reaction mixture containing 100 ng DNA, 200 μM of each dNTP, 200 pmol of each primer, 1.25U of high-fidelity PCR enzyme mix (Fermentas Life Science, St. Leon-Rot, Germany), and 1 × high-fidelity PCR buffer with 1.5 mM MgCl₂. The PCR cycles consisted of denaturation process at 94 °C for 20 seconds, annealing process at 58 °C for 20 seconds, and extension process at 72 °C for 2 minutes, and after the last cycle, samples were incubated at 72 °C for 10 minutes to fill-in the protruding ends of the PCR products. The PCR products were then sequenced by an ABI BigDye Terminator (version 3.1) cycle sequencing ready reaction kit and an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. We directly compared the paired mtDNA sequences from lesional and perilesional skin tissues in patients with As-BD. Any DNA sequence that differed between the two was considered a somatic mutation.

Statistical analysis

Statistical analysis was performed by using the SigmaPlot, version 12.0 (SPSS, Chicago, IL); and the Microsoft Excel 2010 (Microsoft, Redmond, WA). The data are presented as means ± SD of the results obtained from three independent experiments. The significance level of the difference between the control and the experimental groups was determined by the Student’s t-test, and the difference was considered significant when P value was < 0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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Author contributions

CHL defined disease phenotype, collected skin samples, performed keratinocyte work, immunohistochemistry, and drafted the manuscript. SBW measured mtDNA damage and performed the assay for oxidative stress and analysis for the expression of genes of interest by RT-PCR and western blots. YHW designed and supervised the measurement of oxidative stress and determination of sequence variations and oxidative damage of mtDNA in arsenical cancers. HSY held the initial idea of mitochondrial abnormalities in arsenical cancers and supervised the dermatological studies.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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