Neoplastic Transformation of Human Small Airway Epithelial Cells Induced by Arsenic

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INTRODUCTION
Arsenic is a trace element found naturally in the environment. Trace elements are of particular interest given that the levels of exposure to them are potentially modifiable (1). Although inorganic arsenic has been recognized as a human carcinogen for more than a hundred years (2), scientists have been unable to elucidate its carcinogenetic mechanisms. Arsenic is a naturally occurring metalloid, and humans are exposed through contaminated soil, food, and water (3). In addition, occupational exposure occurs mainly through inhalation of airborne particles derived from semiconductor and glass manufacturing or power generation by the burning of arsenic-contaminated coal (2,4,5). Arsenic can be classified into two types, inorganic and organic (6). The former arsenical species are metabolized by methylation to form monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in vivo, which are also carcinogenic.

Epidemiological studies have shown that chronic exposure to arsenic results in liver injury, peripheral neuropathy, keratosis, and increased incidence of cancer of the lung, skin, bladder, and liver (7). In addition, occupational exposure to arsenic increases lung cancer incidence among smokers and underground miners, suggesting a synergistic interaction of arsenic with tobacco smoke or radon found in mines (8,9).

Models of inorganic arsenic-induced carcinogenesis are crucial for a better understanding of the underlying carcinogenic process as well as for prevention and treatment of the disease. In fact, inorganic arsenic is one of the few human carcinogens that do not induce tumors in laboratory animals readily, except at extremely high doses that are irrelevant to human exposure conditions (13,14). In the absence of adequate animal models, in vitro studies become particularly important in assessing the carcinogenic mechanisms of arsenic. Arsenic and arsenical compounds can induce morphological transformants in Syrian hamster embryo and murine C3H 10T1/2 cells (15,16). These agents are also potent clastogens both in vivo and in vitro, as they can induce sister chromatid exchanges.

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and chromosome aberrations in both human and rodent cells (13,15,17).

Arsenical compounds have also been shown to induce gene amplification, arrest mitosis, inhibit DNA repair, and increase c-fos and heme oxygenase expression in mammalian cells (18-20). In addition, arsenic and its derivatives have been implicated as promoters and comutagens for a variety of other agents (21). There is evidence that arsenic can promote cell transformation in vitro. For example, exposure to arsenite of mouse skin epidermal cell line JB6 Cl41 induced anchorage-independent growth in soft agar (22). Exposure of human lymphocytes to low concentrations of arsenite (0.8-10 µM) or to the pentavalent arsenate (2-10 µM) over a range of treatment periods from 1 h to 6 days resulted in enhanced blast transformation and DNA synthesis (23). Moreover, arsenite (0.5 µM) induced malignant transformation in rat liver epithelial TRL 1215 cell line, and these cells produced metastatic tumors upon injection into nude mice (24).

Previous human epithelial cell lines used for arsenic transformation and carcinogenesis study were established by incorporating a virus and other genes to immortalize the cells (25-27). It is possible that these factors affected the genomic stability and intracellular signaling in the cells, which may facilitate increased transformation. Human telomerase reverse transcriptase (h-TERT)-immortalized human small airway epithelial cells, which were established in our lab, have the characteristics of normal cells (28). In this study, we used this cell line to study the ability of inorganic arsenic to induce transformation in culture as an in vitro model of carcinogenesis.

MATERIALS AND METHODS

Cell Culture

The present study used h-TERT immortalized human small airway epithelial cells established in our lab as described (2). Control cells were cultured in SAGM medium supplemented with various growth factors supplied by the manufacturer (SAGM SingleQuots, cat. no. CC-4124; Cambrex Bio Science, Walkersville, MD, USA) and maintained at 37°C in a humidified 5% CO2 incubator. The experimental group consisted of the control cells cultured in the presence of sodium arsenite (0.5 µg/mL) for up to 28 weeks (designated as SAEC-A0.5).

Growth Kinetics Assay and Plating Efficiency

To determine the effect of arsenic on growth rate and saturation density, SAEC and SAEC-A0.5 cells plated at a density of 2 x 10^5 cells from exponentially growing cultures were replated in 60-mm diameter dishes. At each time point studied, triplicate dishes from each group were trypsinized, and total number of cells per dish was determined. Plating efficiency (PE), a measure of the number of colonies originating from single cells, was used for determining the effects of arsenic treatment. PE is determined by the following formula: PE (%) = (number of colonies counted/number of cells inoculated) x 100.

Anchorage-Independent Assay

To test for soft-agar colony growth capacity, SAEC and SAEC-A0.5 cells were plated at a density of 1 x 10^5 cells in 1 mL 0.35% agarose over a 0.7% agar base in a 24-well culture plate. Cultures were fed every three days, and colonies with >50 cells were scored after 4 weeks under a dissecting microscope.

Determination of Frequency of PALA-Resistant Colonies

Exponentially growing cells were plated into 100-mm diameter dishes at a density of 2 x 10^5 each in medium containing N-(phosphonoacetyl)-L-aspartate (PALA) (Drug and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute). PALA was used at 180 µM, which corresponded to nine times the LD_{50} of the cells (LD_{50} for SAEC-A0.5 is 20 µM; data not shown). Cultures were maintained in the selective medium for 12-14 days with fresh medium replenished every three days. They were then fixed and stained, and the number of PALA-resistant colonies was counted. The frequency of resistant colonies was calculated as the ratio of plating efficiency in the presence or absence of PALA.

Micronucleus Assay

Briefly, for the micronucleus assay, cells were incubated with cytochalasin-B (3 µg/mL/L) for 24 h (24). After a hypotonic shock in 0.075 M KCl, cells were stained with acridine orange and examined with a fluorescence microscope under UV light. Cells were counted in three independent experiments involving 1000 cells. The results are plotted as number of micronuclei per 1000 binucleated cells.

Cell Invasion Assay

Invasiveness was measured as described by the vendor (BD BioCoat Matrigel invasion chamber, cat. no. 354480; BD Biosciences, Bedford, MA, USA). Briefly, 8-mm-diameter filters (8 µm pore) of cell culture inserts coated with matrigel were placed in 24-well culture plates. Exponentially growing cells were trypsinized, harvested, and suspended in SAGM medium without supplemental growth factors, and passed repeatedly through a 25-gauge needle to produce a single-cell suspension. After determination of cell count and viability in a hemocytometer by the trypan blue exclusion test, the cells were added to the upper compartment of the chamber (1 x 10^5 cells/chamber). Growth factors added in the medium (complete medium) were used as chemoattractant and placed in the lower chamber. After incubation for 24 h at 37°C in a 5% CO2 incubator, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab, and the cells that had traversed the matrigel and attached to the lower surface of the filter were studied. The filters were fixed, stained with Diff Quick (Sigma, St. Louis, MO, USA), cut out, and mounted on glass slides. The three fields of cell numbers that crossed the membrane were counted under a light microscope and the average value was calculated.
were performed three times with four chambers/cell line.

**In Vitro Healing Wound Assay**

For wound assays, the h-TERT cells were cultured in SAGM medium in the presence or absence of supplemented media with various growth factors supplied by the manufacturer (SAGM Single-Quots, cat. no. CC-4124; Cambrex Bio Science) and maintained at 37°C in a humidified 5% CO₂ incubator. Cells were seeded at a high density, 12 x 10⁴ cells on fibronectin coated coverslips, and wounded one day later when the cells formed a confluent monolayer. The wound was made by scraping with a yellow tip through/ across the cell monolayer. The wound was examined after 18 h.

**Flow-Cytometry Studies**

Cells were trypsinized, washed with PBS, and stained for 30 min on ice with primary antibodies, α₂ or β₄ integrins (BD Biosciences, San Jose, CA, USA), and then with secondary antibodies (Molecular Probes, Carlsbad, CA, USA). Surface expression of integrin receptors on arsenic-treated SAEC cells as well as controls was analyzed by flow-cytometric analysis of surface expression of these two endogenous integrins, the laminin receptor and the collagen receptor in arsenic-treated SAEC cells as well as controls determined by staining with anti-α₂ and anti-β₄ antibodies, respectively.

**Lammelipodia Detection**

Briefly, for lamellipodia detection, arsenic-treated and nontreated cells were washed, trypsinized, washed again, and added to fibronectin-coated coverslips (1 µg/mL) (Sigma) at 37°C for 1 h in DMEM, 2% bovine serum albumin, and 0.5% serum and incubated at 37°C for 30 min. After fixation (PBS, 0.5% Triton X-100 for 5 min). Cells were stained for F-actin with rhodamine-conjugated phalloidin (Invitrogen, San Jose, CA, USA) for 30 min at 37°C, and images were captured using a laser confocal microscope (Nikon, Tokyo, Japan).

**Determination of Protein Expression by Immunocytochemistry Coupled with Confocal Microscopy**

Exponentially growing control and treated cells were plated on a glass chamber slide (Nunc, Naperville, IL, USA) as described (29). Cells were plated at a density of 1 x 10⁴ in 1 mL medium and allowed to grow for two to three days until they reached 70% confluence. The cells were incubated with 1% H₂O₂ in methanol for 30 min to block endogenous peroxidase, washed twice with buffer solution, and fixed with buffered paraformaldehyde in PBS (pH 7.4) at room temperature. Cells were covered with normal horse serum for 30 min at room temperature and incubated with the corresponding primary antibodies at a 1:500 dilution overnight at 4°C. The c-myc, c-fos, and c-Ha-ras (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were all mouse monoclonal antibodies. After washing in buffer solution, cultures were incubated for 60 min at room temperature with antiserum Mouse-Rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at a 1:1000 dilution. After several washes of 5 min each with buffer solution, slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Controls included cultures stained with either the primary or secondary antibodies alone to monitor background staining. Cells were quantified as described (30) and viewed on Zeiss Axioplan TV microscope (Carl Zeiss, Thornwood, NY, USA) using a 40× (11.3 NA) objective lens equipped with a laser scanning confocal attachment (LSM 410; Carl Zeiss).

To excite the Rhodamine secondary antibody, the fluorescent images were collected by an argon/krypton mixed-gas laser (488 nm). Fluorescent images were collected in black and white and changed to red color using Photoshop software, version 5.0. Composite images were quantified using Photoshop. Protein expression of the control and transformed cells was determined by a semi-quantitative estimation based on the relative staining intensity. This computer program gives the area and the intensity of the staining of the cells present in the culture dishes. The experiments were repeated with three similar passages. Standard errors of mean are shown in the different figures. The number of immunoreactive cells was counted in five randomly selected microscopy fields per sample. Statistical analysis was done with the ANOVA F test (randomized block) and comparisons between groups with the orthogonal t test with significance value of <0.05.

**Western Blot Analysis**

Protein was extracted by lysing cells in extraction buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 1 mM phenylmethylsulfonyl fluoride). The protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equivalent amounts of protein (30 µg) were fractionated by electrophoresis in SDS-polyacrylamide gel. The protein was subsequently transferred to PVDF membranes. The secondary antibodies (antirabbit or antimouse) (Amersham Biosciences, Piscataway, NJ, USA) were conjugated to horseradish peroxidase (dilution 1:5000 to 1:10,000). Signals were detected using the ECL system (Amersham Biosciences).

**RESULTS**

The h-TERT immortalized SAEC cells grew as a contact-inhibited monolayer with a population doubling time of ~24 h. At confluence, these cells had a saturation density of ~1.19 x 10⁵ cells/cm² dish (Figure 1A). The cells were treated continuously with sodium arsenite at 0.5 µg/mL for approximately 28 weeks, fresh medium was replenished weekly, and changes in growth kinetics and other transformed phenotypes were monitored periodically over a period of months. The population doubling time of the SAEC-A0.5 cells was similar to that of control
SAEC cells, yet their saturation density increased to $1.57 \times 10^5$ cells/cm$^2$, suggesting that arsenic-exposed cells were able to partially overcome contact inhibition, a characteristic of transformed cells. Furthermore, SAEC-A0.5 cells had a much higher plating efficiency than SAEC cells (Figure 1B), $0.123 \pm 0.028$ in control versus $0.28 \pm 0.053$ in arsenic-treated cells, $P < 0.01$. Anchorage independent growth usually correlates strongly with invasiveness in many cell types. Our data demonstrated that only SAEC-A0.5 cells formed agar-positive clones, with a rate of >5% (Figure 1C). In contrast, control SAEC cells showed no anchorage-independent growth. Soft agar positive clones from arsenic-treated cells were isolated and used for additional experiments to assess transformation capabilities.

Gene transformation is a frequent manifestation of genomic instability in cancer cells. Using resistance to the chemotherapeutic drug PALA as an index of gene amplification, our results demonstrated that SAEC-A0.5 cells exhibited a much higher frequency of PALA resistance than control SAEC cells. The incidence of PALA resistance was $1.3 \times 10^3$ in SAEC cells. In arsenic-treated cells, the frequency was $1.2 \times 10^2$, a $10^3$-fold increase in activity of CAD gene. These data indicate that arsenic exposure induced genomic instability in the SAEC cells during the neoplastic transforming process (Figure 1D).

Number of micronuclei is a conventional measure of genotoxicity and therefore used in this study to analyze arsenic-induced genotoxicity. Our results showed a 4.8-fold increase in the incidence of micronuclei in SAEC-A0.5 cells compared with control cells (Figure 1E and F). The incidence was substantially increased to more than 109 micronuclei per 1000 binucleated cells in SAEC-A0.5 cells, in contrast to only 27 micronuclei per 1000 binucleated cells in SAEC control cells (10% versus 3%), suggesting that chronic arsenic treatment induced genomic instability in SAEC cells.

Figure 2A shows invasive characteristics of control and arsenic-treated SAEC...
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cells when scored 23 h after plating onto matrigel basement membranes in Boyden chambers. Growth factors were used as chemoattractant. The number of cells that migrated through the membrane increased from 69.3 ± 18.4 in control to 149.3 ± 35.6 in arsenic-treated cells, \( P < 0.05 \), more than double, a clear indication that arsenic treatment resulted in an increase in invasive capabilities. Another indicator of transformation in arsenic-treated cells was in vitro wound-healing (Figure 2B), which is generally used for assessing cell motility. Arsenic-treated SAEC cells exhibited much greater cell migration ability in response to growth factors than control cells.

Because change in cell adhesion characteristics may imply change in integrin function, which plays an essential role in cell attachment, we examined the expression of various integrin receptor functions in control and arsenic-treated SAEC cells. Flow cytometry indicated that \( \alpha_2 \) integrin did not show any difference between control and arsenic-treated cells (Figure 2C). Similar results were also found in \( \alpha_3, \alpha_5, \alpha_1, \) and \( \alpha_\beta_3 \) integrins (data not shown). However, \( \beta_4 \) integrin protein expression was lost in arsenic-treated cells while its expression was similar in control cells. Figure 2D shows lamellipodia formation in control and arsenic-treated cells. The arsenic-treated SAEC group had a higher percentage of lamellipodia formation than control cells (Figure 2D), 75 ± 8 versus 28 ± 10, \( P < 0.01 \); moreover, larger lamellipodia were formed in arsenic-treated cells than controls, indicating an alteration in epithelial morphology and attachment to the fibronectin substrate upon plating, both of which contribute to the process of transformation as seen in representative cells (Figure 2E).

Malignant transformation is frequently associated with changes in expression of specific oncogenes and tumor suppressors. Immunofluorescence studies indicated that there was a significant increase in c-myc, c-Ha-ras, and c-fos protein expression in arsenic-treated cells in comparison with control cells (Figure 3A). The expression of c-myc, c-fos, and c-Ha-ras was increased approximately up to 3-, 3.1, and 2.6-fold, respectively. Representative images of c-myc, c-Ha-ras, and c-fos cells are shown in Figure 3B.

Changes in c-Ha-ras, c-myc, and c-fos protein expression were confirmed by Western blot analysis in control and SAEC-A0.5 cells (Figure 4A and B). Phosphorylation of ERK and retinoblastoma in random growth conditions was examined by Western blot between SAEC and SAEC-A0.5 cells, and these changes were...
further confirmed by examining one of the soft-agar clones of SAEC-A0.5 cells, which showed even higher expression of c-fos and c-jun and more intensive phosphorylation of ERK (Figure 4A, B, and C). Under these culture conditions, the Rb and ERK proteins were hyperphosphorylated in SAEC-A0.5 cells, whereas the control cells maintained a basal level of phosphorylation. These results demonstrated that expression of molecules controlling cell proliferation and transformation, such as c-myc, c-Ha-ras, and c-fos, were altered by chronic arsenic treatment. Wild-type p53 exhibited a significant decrease in SAEC-A0.5 cells compared with SAEC (Figure 4D). However, the coding region of p53 in SAEC-A0.5 cells revealed no change in sequencing (data not shown).

DISCUSSION

The metalloid arsenic, a naturally occurring element, is a serious global environmental concern and a severe health problem affecting millions of people worldwide. To have an effective interventional approach in treatment and prevention of arsenic-induced carcinogenesis in vitro, a model of arsenic transformation assays based on human epithelial cells is needed. The objective of this study was to analyze whether arsenic was able to transform lung cells, based on the h-TERT immortalized human small airway epithelial cells previously described (2).

Under these culture conditions, the Rb and ERK proteins were hyperphosphorylated in SAEC-A0.5 cells, whereas the control cells maintained a basal level of phosphorylation. These results demonstrated that expression of molecules controlling cell proliferation and transformation, such as c-myc, c-Ha-ras, and c-fos, were altered by chronic arsenic treatment. Wild-type p53 exhibited a significant decrease in SAEC-A0.5 cells compared with SAEC (Figure 4D). However, the coding region of p53 in SAEC-A0.5 cells revealed no change in sequencing (data not shown).

Micronuclei are a reliable cytogenetic marker of chromosomal instability based on the presence of nuclear fragments in binucleated cells (29,31). In our study, the incidence of micronuclei in SAEC-A0.5 cells was >10%, in contrast to the 3% incidence in SAEC (Figure 1E), suggesting that chronic arsenic treatment induced genomic instability in the human small airway epithelial cells.

Transformation/carcinogenesis is a multistage process. The importance of maintaining genomic stability is evidenced by the fact that transformed cells often contain a variety of chromosomal abnormalities such as aneuploidy, translocations, and inversions. Genomic instability induced by chemicals or drugs may contribute to transformation by accumulating genetic changes that ultimately lead to malignant conversion. Our results based on micronuclei formation and the PALA assay indicated that arsenic resulted in genomic instability in the h-TERT immortalized human epithelial cells, consistent with previous studies in different cell types. The PALA assay is designed to measure directly the frequency with which gene amplification occurs in mammalian cells in culture (32,33). PALA prevents de novo pyrimidine biosynthesis through inhibition of the trifunctional enzyme CAD (carbamyl phosphate synthetase/aspartate transcarbamylase/dihydro-orotase), an essential enzyme in the synthesis of UMP (34). When cells are exposed to PALA, the synthesis of pyrimidines is inhibited, resulting in DNA mutations or chromosomal breaks (35). If PALA treatment is maintained, cells eventually undergo apoptosis (36). Cells become resistant to PALA through amplification of the CAD gene (37), yet the saturation density substantially increased in SAEC-A0.5, suggesting that this phenotype may represent an early manifestation of transformation. The increased plating efficiency indicated a portion of highly proliferating cells that boosted the cell growth advantage and may be responsible for the loss of contact inhibition of growth and increased saturation density in the transformed cells.
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which raises the cellular concentration of the CAD enzyme to overcome the inhibitory effects of the drug. Therefore, the frequency of the cells developing PALA resistance provides an objective measure of those cells undergoing gene amplification, which can be used as an indication of genomic instability.

Invasion capability of cells has been shown to correlate with their malignant characteristics in vivo (30). Transformed cells have enhanced invasive ability in vitro, which induces alterations in phenotype. In particular, arsenic-transformed cells displayed anchorage-independent growth as well as enhanced migration and invasive ability, suggesting that SAEC-A0.5 cells acquired some characteristics of oncogenic transformation through long-term arsenic treatment.

Accumulating evidence demonstrates that malignant transformation is associated with changes in the repertoire of expression of the integrin family of molecules, which mediate cell-matrix and cell-cell interactions. In the present study, we analyzed the presence of several major integrins on the cell surface of arsenic-treated cells versus the non-treated SAEC cells. Results indicated that α2 integrin, along with α3, α5, α1, and αβ3 integrins, did not show much difference between control and arsenic-treated cells. However, β4 integrin protein expression was lost in arsenic-treated cells, indicating a change in the extracellular matrix receptor affected by the chemical used. Lamellipodia formation was found in both control and experimental cells. The arsenic-treated SAEC cells had a higher percentage of lamellipodia formation than control cells, indicating a more potent protractive force on the fibronectin substratum; this feature is possibly associated with the lost expression of β4 integrin.

Altered expression of genes such as oncogenes or tumor suppressors is likely affected by mutagens such as chemicals or drugs, and thus contributes to oncogenic transformation (38,39). Thus c-Ha-ras, c-myc, and c-fos have been used as prominent markers for malignant transformation in many cell types. Their upregulation leads to activation of several major signaling pathways in transformed or tumor cells, particularly in some types of arsenic-transformed cells (40-43). The change in expression of such proteins supported our hypothesis that, after a long period of treatment with arsenic, the cells exhibited characteristics of malignant transformation. Hence they was used in the present study to define cell transformation of arsenic-treated cells.

p53 plays a critical role in maintaining genome integrity and accuracy of chromosome segregation. The effects of arsenic on p53 activation have been extensively studied and yet inconsistent. Some reports demonstrated that arsenic could induce p53 accumulation in an ATM-dependent pathway (44); the overexpressed p53 was a mutant form, and most frequent mutation sites were found in exons 5 and 8, so mutated p53 subsequently led to its normal function loss. Other studies (45-47) documented that the promoter of p53 in arsenic-exposed cells exhibited hypermethylation as some carcinoma appeared; consequently, p53 expression decreased, resulting in its function loss or partial loss, so the cells were predisposed to uncontrolled proliferation, genomic instability, or apoptosis.

In our study, p53 did not show mutations in its coding region upon chronic arsenic...
treatment, yet its protein expression was remarkably decreased relative to the control cells. Because p53 is critical in maintaining genomic stability, this finding emphasizes many of the changes described in this study.

The benefits that this study provides include the technology needed to define signs of malignant transformation caused by arsenic that may lead to the design of specific tools for diagnosis, treatment, or prevention of lung cancer. The information presented here-related to protein expression of early oncogenes such as c-myc and c-fos, oncogenes such as c-Ha-ras, and a tumor suppressor gene such as p53-will help to define the damage related to transformation induced by arsenic exposure.

Studies of populations exposed to high levels of arsenic in drinking water have raised the possibility of a causal relationship for lung cancer. Marshall et al. (12) provided additional evidence linking arsenic to lung cancer. Our work used micronuclei as a biomarker in a h-TERT immortalized cell line that can be used for human lung cancer research. Arsenic-induced cell transformation was associated with genomic instability through analysis of micronuclei formation. A 4.8-fold increase in micronuclei incidence in arsenic-treated cells was detected. The arsenic-treated SAEC cells also presented a higher percentage of lamellipodia formation than control cells, enhanced by the lost expression of β4 integrin and in conjunction with increased PALA-resistant characteristics.

Human epithelial cell lines used for arsenic transformation and carcinogenesis studies have been previously established to immortalize the cells by incorporating a virus and other genes. It is possible that these factors affect the intracellular signaling in these cells, which may facilitate transformation. To a greater understanding of lung cancer, the arsenic-induced gene expression changes have been shown in prior work by many groups. Therefore, the novelty of this study was to use a more normal human lung cell line than previously reported. The h-TERT immortalized human SAEC is generally genomically stable, taking much longer for such cells to be transformed, compared with other methods that use virus and T-antigen to immortalize the cells. Such cells had certain characteristics consistent with a cancer cell phenotype.

The novelty of these data is the use of an established immortalized h-TERT human small airway epithelial cell line that has the characteristics of normal cells. Thus, this work provides a combination of phenotypic and molecular tools to characterize the carcinogenic process induced by arsenic. Taken together, our results strongly suggest that malignant transformation occurred in the h-TERT immortalized human small airway epithelial cell after inorganic arsenic treatment in vitro.

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