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Clonal variability in chromosomal instability as a potential driver in the acquisition of tumorigenic phenotype in chronic arsenic-exposed and hsa-miR-186 overexpressing human keratinocytes

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Abstract

Chronic arsenic exposure through drinking water is a global health issue, affecting more than 200 million people. Arsenic is a group I human carcinogen and causes chromosomal instability (CIN). Arsenic exposure is the second most common cause of skin cancer after UV radiation. hsa-miR-186 is overexpressed in arsenic-induced squamous cell carcinoma relative to premalignant hyperkeratosis. Among predicted targets of hsa-miR-186 are cell cycle regulators including regulators of mitotic progression. Disruption of mitotic progression can contribute to CIN. Thus, we hypothesized that hsa-miR-186 overexpression contributes to malignant transformation of arsenic exposed HaCaT cells by induction of CIN. Stable clones of HaCaT cells transfected with pEP-hsa-miR-186 expression vector or empty vector were maintained under puromycin selection and exposed to 0 or 100 nM $NaAsO_2$ and cultured for 29 weeks. HaCaT clones overexpressing hsa-miR-186 and exposed to $NaAsO_2$ showed increased CIN and anchorage independent growth at 29 weeks in a stochastic manner, in contrast to unexposed empty vector transfected clones. These results suggest that clonal variability mediates arsenic-induced carcinogenesis in hsa-miR-186 overexpressing human keratinocytes.

Keywords

arsenic; chromosomal instability; miRNA dysregulation; clonal variability; human keratinocytes; aneuploidy; carcinogenesis

Introduction

Arsenic is a serious public health concern and arsenic poisoning is the cause of many fatal diseases (Paranjape, Slack et al. 2009). Underground drinking water is the major source of human arsenic exposure (Paranjape, Slack et al. 2009). Globally, over 200 million people consume drinking water contaminated with high levels of arsenic (Paranjape, Slack et al.

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2009). This problem occurs not only in third world and developing countries but also in the U.S.A. Approximately, 2.9 million people in the U.S. consume arsenic-contaminated drinking water (Martinez, Vucic et al. 2011). The United States Environmental Protection Agency and the World Health Organization have regulated the arsenic content of public drinking water to <10 ppb. However, arsenic content in private supplies, e.g. rural well water is not regulated in the U.S.A., and many have high arsenic levels (Martinez, Vucic et al. 2011). The long-term effects of arsenic exposure include the manifestation of different malignant and nonmalignant diseases, like skin, lung and bladder cancers, cardiovascular and neurodegenerative diseases.

Arsenic is a group I human carcinogen, but the mechanism of arsenic-induced carcinogenesis is not established. Arsenic has been characterized as a clastogen because it causes chromosomal instability (CIN) both *in vitro* (Taylor, McNeely et al. 2008) and in *vivo* (Hanlon and Ferm 1986, Ochi, Kita et al. 2008). However, the molecular etiology of arsenic-induced CIN remains unclear. Skin is the primary target organ of arsenic toxicity and arsenic exposure is the second most common cause of skin cancer, after UV radiation. Specifically, arsenic-induced squamous cell carcinoma (SCC) is the most common cancer caused by chronic arsenic poisoning (Sarma 2016). SCC is a malignant form of cancer with high potential for metastasis that originates from keratinocytes. The premalignant lesion for arsenic-induced SCC is the hyperkeratotic (HK) nodule. HK is a typical cutaneous manifestation of chronic arsenic poisoning. We analyzed samples of known arsenic-induced premalignant lesions (HK) and SCC to determine the potential microRNA changes associated with the progression to carcinoma (Al-Eryani, Jenkins et al. 2018). We found higher hsa-miR-186 expression in malignant SCC lesions compared to HK (Al-Eryani, Jenkins et al. 2018) and when hsa-miR-186 is overexpressed in human keratinocytes, it caused CIN (Wu, Ferragut Cardoso et al. 2019). Bioinformatic analysis using the DIANA-miRPath tool revealed that hsa-miR-186 targets proteins that play a key role in the establishment of the mitotic spindle checkpoint and chromosome segregation (Wu, Ferragut Cardoso et al. 2019).

The current study investigates the effect of chronic concurrent arsenic exposure and hsa-miR-186 overexpression, simulating the conditions in people chronically exposed to arsenic. We sought to delineate karyotypic changes with particular interest on chromosomal translocations and other structural abnormalities. Our focus on the karyotypic changes induced by arsenic exposure and hsa-miR-186 overexpression will elucidate the kind of the chromosomal instability that contributes to the stochastic process of carcinogenesis. Our proposed mechanism could also apply to other internal cancers caused by arsenic, rendering hsa-miR-186 overexpression a potential biomarker of arsenic-induced carcinogenesis.

Materials and methods

HaCaT cell culture and arsenite exposure

Human immortalized keratinocytes, HaCaT cells were the kind gift of the Dr. TaiHao Quan (University of Michigan Ann Arbor). The identity of the cells was confirmed at the beginning, middle and end of the experiment by STR (Short Tandem Repeat) mapping (Genetica DNA Laboratories/LabCorp, Burlington, NC) as described previously (Ferragut

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Cardoso, Banerjee et al. 2022). HaCaT cells were cultured in alpha modification of minimal essential medium (α-MEM, Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (characterized, HyClone, Logan, UT, USA), 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (GIBCO, Invitrogen, Carlsbad, CA, USA). HaCaT cells were transfected with pEP-hsa-miR-186 expression vector (Cell Biolabs, Inc., San Diego, CA) or empty vector and maintained with 0.9 μg/mL puromycin (P7255, Sigma-Aldrich, St Louis, MO, USA) (Wu, Ferragut Cardoso et al. 2019). The clones were isolated and characterized for hsa-miR-186 expression (Wu, Ferragut Cardoso et al. 2019). Three hsa-miR-186 transfected clones with the highest expression and three empty vector transfectants with lowest expression were selected for study (Wu, Ferragut Cardoso et al. 2019). The six clones were propagated independently for 8 weeks in complete α-MEM with 0 or 100 nm sodium arsenite ($NaAsO_2$) (CAS 7784-46-5, ThermoFisher Scientific, Waltham, MA, USA) (Wu, Ferragut Cardoso et al. 2019). This concentration of $NaAsO_2$ was selected based on the average blood arsenic level observed in an epidemiological study in China where people used tube wells with high arsenic concentration (Chen, Graziano et al. 2011). Chronic arsenic intoxication and arsenic-induced skin lesions and epidermal cancers were observed in this population (Pi, Diwan et al. 2008). The six clones cultured for 8 weeks were re-established from frozen stocks of cells and further propagated until they reached 29 weeks. The exposure to 0 or 100 nM of $NaAsO_2$ was continued as in the earlier study. Thus, there are twelve cultures total with three maintained under each of the following four different conditions: 1) vector control transfected + 0 nM $NaAsO_2$, 2) vector control transfected + 100 nM $NaAsO_2$, 3) hsa-miR-186 overexpressing + 0 nM $NaAsO_2$, 4) hsa-miR-186 overexpressing + 100 nM $NaAsO_2$. Cultures were grown at 37°C in humidified atmosphere of 95% air and 5% CO_2. Cells were passaged twice per week (every 3–4 days) and a million cells were plated per 100 mm dish at every passage. No mycoplasma contamination was observed after monthly screening with PCR (MycoSensor PCR Assay Kit #302108, Agilent).

HeLa cells

HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle Media) (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) (characterized, HyClone,), 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (GIBCO, Invitrogen). Cultures were grown at 37°C in humidified atmosphere of 95% air and 5% CO_2. Cells were passaged twice per week (every 3–4 days) and a million cells were plated per 100 mm dish at every passage. HeLa cells and HeLa cell lysates were used as positive controls in soft agar assays and Western blots, respectively.

Cytogenetic analysis

HaCaT clones transfected with either empty vector or overexpressing hsa-miR-186 and exposed to 0 or 100 nM of $NaAsO_2$, were seeded into 100 mm dishes with 7 mL complete α-MEM. Cells were allowed to acclimate for 48 h and re-enter normal cell cycle pattern. Cells were arrested at metaphase after addition of demecolchicine solution (#D1925, Sigma) to final concentration of 0.2 μg/mL. The cultures were then incubated at 37°C for 1 h. At the end of treatment, media were collected to include any loosely adherent mitotic cells. After collecting the media, the attached cells in the dish were washed with phosphate buffered

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saline (PBS) and dissociated with 0.25% trypsin in phosphate buffered saline containing 5 mM Na_2-EDTA (disodium ethylenediaminetetraacetic acid). Complete media was added to the trypsinized cells to inactivate the trypsin. The trypsinized cells were then added to the media collected prior to trypsinization (which includes the loosely adherent cells). Then, cells were pelleted by centrifugation at 1000 rpm for 5 min at 4 °C. The pellets were resuspended and incubated in 10 mL of 0.075 M potassium chloride (KCL) hypotonic solution for 17 min to swell the cells and the nuclei. At the end of 17 min, 1 mL of freshly mixed methanol-glacial acetic acid (3:1::v:v) was added to the resuspended cells and cells were collected by centrifugation at 1000 rpm for 5 min at 4 °C. Subsequently, pellets were suspended gradually in 10 mL methanol-acetic acid and incubated at room temperature for at least 20 min, collected by centrifugation at 1000 rpm for 5 min at 4 °C and resuspended in methanol-acetic acid. The cells were dropped onto microscope slides to provide a suitable density of metaphases for microscopic analysis.

Karyotype analysis

Slides were stained for G-banding of chromosomes. After the cells were fixed, slides were immersed in 0.063% trypsin solution for 30–60 sec at pH 7.2. A 2% FBS wash followed to stop the trypsin digestion. Then, the slides were rinsed with pH 7.0 Gurr's buffer, followed by dehydration with 70% and 95% ethanol, and rinsed in pH 6.8 Gurr's buffer. The slides were stained for 3 min with 5% Giemsa and coverslipped with Cytoseal™-60. Karyotypes were determined in twenty metaphases for each clone at 9 and 29 weeks timepoints. Metaphases were analyzed using an Olympus light microscope and karyotypes were assembled using Applied Spectral Imaging software (ASI, Carlsbad, CA).

Soft agar colony formation assay

Suspension of 3% LMP agarose (FMC, Philadelphia, PA, USA) in PBS was prepared, autoclaved and kept in a liquid state in a 60° C water bath. Media (α-MEM) containing 15 % FBS was prepared and kept at 37° C. Bottom agar containing 1% agarose was made and 2 ml of bottom agar was suspended per well in 6-well plate. Bottom agar was allowed to solidify at room temperature. Then, cells were collected, counted and passed through a cell strainer to make sure that single cells were plated. The top agar containing 0.4% agarose was prepared and kept at 37° C. The required volume of cell suspension was added on the top agar. The density of cells that were plated was: 30,000 HaCaT cells/well and 10,000 HeLa cells/well as positive control. Three technical replicates were used for each clone. Top agar containing cells (2 ml) were plated per well in a 6-well plate. The plates were put at 4° C for 20 min, followed by 2–3 week incubation at 37° C. After 2–3 weeks, the top media was taken out, MTT staining solution (5 mg/mL MIT in media containing 10% FBS, 500 μl for each well), was added and stained for 1–2 hours at 37 °C. Colonies were then identified and counted microscopically.

Western blot analysis

Cells were lysed with a solution of 10 mM Tris-HCl pH 7.4, 1 mM Na_2EDTA, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate (Na_3VO_4) and 1x protease inhibitor cocktail (Complete, Roche, Mannheim, Germany). Insoluble debris was removed from lysates after sonication by centrifugation

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at 13,000 × g, at 4 °C for 15 min. Protein concentrations were determined with a bicinchoninic acid (BCA) assay (ThermoFisher Scientific). Aliquots of 20 μg of total protein extracts were resolved by electrophoresis in 4–20% Criterion™ TGX™ Precast gradient sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Bio-Rad). The resolved proteins were transferred onto polyvinylidene fluoride membrane (PVDF, Millipore). Staining with Coomassie Brilliant Blue R250 (ThermoFisher Scientific) followed, to ensure equal loading and transfer of proteins. Membranes were then blocked in 5% milk in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 h. The membranes were subsequently probed with antibodies against slug (#9585, Cell Signaling, 1:2000 in 1% milk in TBSTS), n-cadherin (#13116, Cell Signaling, 1:2000 in 1% milk in TBST), ZO-1 (#8193, Cell Signaling, 1:2000 in 1% milk in TBST), β-catenin (#8480, Cell Signaling, 1:2000 in 1% milk in TBST), e-cadherin (#3195T, Cell Signaling, 1:3000 in 1% milk in TBST), claudin 1 (#13255, Cell Signaling, 1:1000 in 1% milk in TBST) and GAPDH (#5174S, Cell Signaling,1:1000 in 5% milk in TBST. Blots were then incubated with anti-rabbit (#7074, Cell Signaling Technology, 1:1000 in 1% milk in TBST) or anti-mouse (#7076, Cell Signaling Technology, 1:1000 in 1% milk in TBST) HRP-conjugated secondary antibodies as appropriate. Restore™ Western Blot Stripping Buffer (#21059, ThermoFisher Scientific) was used for stripping between probing with different antibodies. For the detection of signals, blots were incubated with an enhanced chemiluminescent substrate (ECL) (ThermoFisher Scientific). Images were acquired using FOTO/Analyst FX. Densitometry analysis was performed using Image Studio Lite Software version 5.2.5.

Statistical analysis

Data were analyzed with two-way ANOVA and Tukey's post-hoc test (for multiple comparisons). Three-way ANOVA was used to analyze chromosomal instability data over the course of time. Data are expressed as mean +/− SD (Standard Deviation). The level of significance is established at p value   0.05. GraphPad Prism 10.0.1 software (GraphPad Software, San Diego, CA, USA) was employed for all statistical analyses and generation of bar graphs.

Results

Chronic culturing of human keratinocytes exacerbates their intrinsic CIN

Chromosomal instability is a hallmark of carcinogenesis and arsenic causes CIN both *in vitro* and *in vivo* (Council 2001). We showed in an earlier publication (Wu, Ferragut Cardoso et al. 2019)that human HaCaT Keratinocytes overexpressiong miR-186 exhibited increased CIN that was exacerbated when the cells were also exposed to 100 nM $NaAsO_2$ for 8 weeks. Thus, we wanted to screen for specific structural and numerical chromosomal abnormalities that could drive the transformation process of HaCaT overexpressing hsa-miR-186 and/or exposed to 100 nM $NaAsO_2$. For this purpose, we performed karyotypic analysis at 9 and 29 weeks of culture, as described in methods. HaCaT cells have background levels of chromosomal aberrations (Boukamp, Petrussevska et al. 1988). HaCaT cells are hypotetraploid with a range of 72–88 chromosomes, with the presence of marker chromosomes M1: t(3q4q), M2: i[9q], and M4: [4p18q] (Boukamp, Petrussevska et al. 1988). We validated the authenticity of the cell line by observing the presence of marker

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chromosomes in addition to the STR mapping. Since HaCaT cells exhibit intrinsic levels of chromosomal instability, we compared our karyotypes with the normal human karyotype (46 chromosomes). We analyzed twenty metaphases for each of the three vector control transfected clones exposed to 0 or 100 nM sodium arsenite and each of the three hsa-miR-186 overexpressing clones exposed to 0 or 100 nM sodium arsenite. Analysis of the chromosomal abnormalities showed that all clones exhibited structural and numerical chromosomal abnormalities and the CIN levels varied among clones of the same group. We counted the total number of supernumerary chromosomes and structural chromosomal abnormalities at 9 and 29 weeks and tested the effect of arsenic exposure, hsa-miR-186 overexpression and time. We observed a significant effect of time (p=0.0018) for the acquisition of both structural chromosomal abnormalities and supernumerary chromosomes, suggesting that chronic culturing exacerbates HaCaT cells' intrinsic chromosomal instability (Figure 1).

We also observed that there is clonal variability in the acquisition of either structural chromosomal instability or supernumerary chromosomes, since different clones of the same group exhibit different levels of abnormalities (Figure 1). The arsenic exposed hsa-miR186 overexpressing HaCaT cells tend to have increased structural chromosomal instability (Figure 1, Panel B), however this is not statistically significant due to high standard deviation, caused by clonal variability. Clonal variability is a fundamental principle in cancer biology [10] and could mediate chronic arsenic-induced carcinogenesis in hsa-miR-186 overexpressing keratinocytes.

Arsenic exposed and hsa-miR-186 overexpressing HaCaT cells exhibit structural chromosomal abnormalities in a stochastic manner.

We observed that HaCaT cells overexpressing hsa-miR-186 and exposed to arsenite exhibited structural abnormalities more frequently compared to all other cells, at both 9 and 29 weeks. Therefore, we performed detailed analysis of karyotypes to determine which structural abnormalities frequently occurred. These abnormalities included deletion of chromosomal material in chromosome 1p, extra chromosomal material of unknown origin in chromosomes 11p and 20q, presence of double minute chromosomes and increased number of marker chromosomes (Figure 2).

Both at 9 and 29 weeks we observed increased structural chromosomal instability for hsa-miR-186 overexpressing clones compared to vector control cells and these chromosomal abnormalities were more pronounced when the cells were also exposed to arsenic (Figure 2). The latter suggests that the combination of arsenic exposure and hsa-miR-186 overexpression causes structural chromosomal instability in human keratinocytes. Arsenic and hsa-miR-186 could have a synergistic role in arsenic-induced carcinogenesis. We also observed that all vector control clones exhibited less variability in these structural chromosomal abnormalities compared to hsa-miR-186 overexpressing clones at both 9 and 29 weeks (Supplemental Figure 1), (Supplemental Figure 2). This result is consistent with our recent observation of increased tetraploidy and aneuploidy in human Ker-CT keratinocytes overexpressing miR-186 (Ferragut Cardoso, Nail et al. 2023) and.suggests that each clone might take a different transformation pathway during arsenic-induced

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carcinogenesis. These variations in structural chromosomal abnormalities that we examined were also more pronounced at 29 weeks compared to 9 weeks (Figure 1, Panel B), (Figure 2, Panel A, Panel B), consistent with a significant effect of time.

These observations propose that chronic arsenic exposure and hsa-miR-186 overexpression cause structural chromosomal aberrations in chromosomes 1, 11, 20 and increase the number of double minute and marker chromosomes, compared to unexposed vector control cells. Overall, both at 9 and 29 weeks, CIN is more pronounced in arsenic exposed and hsa-miR-186 overexpressing HaCaT cells, suggesting a synergistic role of arsenic and hsa-miR-186 overexpression in chromosomal instability, which could contribute to clonal variability of cells during their malignant transformation process over the course of time.

HaCaT cells overexpressing hsa-miR-186 and chronically exposed to arsenic developed anchorage-independent growth.

The ability of cells to proliferate in suspension, unattached to any matrix, is a characteristic of transformed cells, correlating with tumorigenic potential *in vivo*. HaCaT overexpressing hsa-miR-186 and exposed or not to arsenic were tested *in vitro* for anchorage-independent growth in agar (Borowicz, Van Scoyk et al. 2014). The assay was performed at an early time point (9 weeks) and at a later timepoint (29 weeks) to assess if hsa-miR-186 overexpression accelerates the arsenic transformation process. We did not observe any colony formation at 9 weeks but we observed that all HaCaT overexpressing hsa-miR-186 and exposed to arsenite clones formed colonies at 29 weeks. Specifically, there was statistically significant greater colony formation ability in hsa-miR-186 overexpressing and exposed to arsenite clones compared to all other groups (Figure 3).

Examination of markers of epithelial-to-mesenchymal transition (EMT)

The induction of epithelial-to-mesenchymal transition (EMT) is an important mechanism for the progression of carcinomas to a metastatic stage (Kalluri and Weinberg 2009). During EMT, the levels of the epithelial markers are suppressed, and the levels of the mesenchymal markers are induced (Kalluri and Weinberg 2009). We screened empty vector or overexpressing hsa-miR-186 and exposed to 0 or 100 nM of $NaAsO_2$ for the expression levels of epithelial and mesenchymal markers. We determined expression of the epithelial markers e-cadherin, β-catenin, claudin 1, and ZO-1 and mesenchymal markers n-cadherin and slug (Kalluri and Weinberg 2009). After 29 weeks of culturing, Slug expression was significantly increased in cells overexpressing hsa-miR-186 compared to vector control transfected cells regardless of arsenite exposure (Figure 4). All other markers showed not significant changes in expression (Supplemental Figure 3).

We did not observe any statistically significant changes in expression of any epithelial or mesenchymal markers at earlier time points. These observations suggest that HaCaT transfected with hsa-miR-186 start to undergo epithelial to mesenchymal transition sometime by 29 weeks. Nonetheless, the anchorage independent growth demonstrated by clones overexpressing miR-186 and exposed to arsenite indicate that these cultures contain transformed cells. It may be that the transformed fraction of the total is too small to provide statistically increased changes in expression of most of the EMT markers.

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Discussion

Arsenic is a toxic metalloid and chronic arsenic exposure is a serious public health concern and the cause of many fatal diseases (Smith, Lingas et al. 2000), including cancer(Martinez, Vucic et al. 2011). Arsenic is classified as a group I human carcinogen by the International Agency of Research on Cancer (IARC) since 1980 (Kapaj, Peterson et al. 2006). The latter means that there is sufficient evidence of carcinogenicity to humans (Martinez, Vucic et al. 2011). Arsenic is also clastogenic and causes chromosomal instability (CIN) both *in vitro* and *in vivo* (Council 2001). CIN is a hallmark of carcinogenesis, and is associated with poor prognosis, metastasis, and therapeutic resistance (Sansregret, Vanhaesebroeck et al. 2018). However, the molecular mechanism by which arsenic induces CIN-mediated carcinogenesis is yet to be elucidated (Wu, Ferragut Cardoso et al. 2019).

Arsenic causes several cancers, such as lung, bladder, kidney, liver and non-melanoma skin cancer (Martinez, Vucic et al. 2011). Chronic arsenic exposure is the second most common cause of skin cancer, following sunlight (Hunt, Srivastava et al. 2014). Despite evidence in humans, animal models fail to replicate these observations. The lack of an animal model has made it difficult to determine the exact mode(s) of action underlying arsenic-induced carcinogenicity (Martinez, Vucic et al. 2011). However, human immortalized keratinocytes (HaCaT) malignantly transformed by chronic incubation in low concentration of sodium arsenite are a very well-established *in vitro* model to study arsenic-induced skin carcinogenesis (Pi, Diwan et al. 2008, Sun, Pi et al. 2009). Specifically, continuous exposure of HaCaT cells to toxicologically relevant sodium arsenite concentration (100 nM) for 29 weeks induced malignant transformation (Pi, Diwan et al. 2008, Sun, Pi et al. 2009). HaCaT cells are a spontaneously immortalized human keratinocyte cell line with unlimited growth potential which maintains full epidermal differentiation capacity (Boukamp, Petrussevska et al. 1988). They are hypotetraploid with a range of 72–88 chromosomes, including marker chromosomes (M1: t(3q4q), M2: i[9q], and M4:[4p18q] (Boukamp, Petrussevska et al. 1988)]. The presence of these marker chromosomes can be used to validate the authenticity of the cells and exclude cross-contamination with other human cell lines (Boukamp, Petrussevska et al. 1988).

hsa-miR-186 expression was found to be highly elevated in some cases of squamous cell carcinoma induced by chronic arsenic exposure via drinking water, relative to non-malignant hyperkeratosis (States 2015, Al-Eryani, Waigel et al. 2018). hsa-miR-186 plays a crucial role in various biological processes and may act as an oncogenic or tumor-suppressor miRNA depending on context. The likely role of miR-186 in carcinogenesis has been reported and dysregulated miR-186 levels can either promote or inhibit tumorigenesis (Cardoso, Al-Eryani et al. 2018, Su, Zhou et al. 2018).

Upregulated hsa-miR-186 suppresses securin, which is one of its targets (Li, Yin et al. 2013, States 2015). Normally, securin binds to and inhibits a protease called separase, which, when released following securin degradation, is active and cleaves cohesins that hold the sister chromatids together, thus initiating anaphase (Solomon and Burton 2008). Therefore, suppressed securin levels, because of overexpression of hsa-miR-186, would allow anaphase progression and contribute to aneuploidy by promoting premature chromatid separation

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(States 2015). Analysis using the bioinformatic tool DIANA miRPath V3.0 (Vlachos, Zagganas et al. 2015), showed that hsa-miR-186 is predicted also to target mRNAs of other proteins that regulate the cell cycle and are components of the spindle assembly checkpoint (SAC) and anaphase promoting complex (APC), including: budding uninhibited by benzimidazoles 1 (BUB1) and cell division cycle 27, (CDC27)(Vlachos, Zagganas et al. 2015). According to the bioinformatic prediction, hsa-miR-186 targets and suppresses BUB1 and CDC27, which play a role in the establishment of the mitotic spindle checkpoint and proper chromosome segregation. Also, ectopic expression of hsa-miR-186 in HaCaT cells induces a significant increase in numerical and structural chromosomal abnormalities and these aberrations are further increased with chronic arsenite exposure at 8 weeks of culture (Wu, Ferragut Cardoso et al. 2019). Overall, the studies described above, suggest that upregulated levels of hsa-miR-186 cause chromosomal instability and hsa-miR-186 overexpression exacerbates the arsenic-induced chromosomal instability associated with skin carcinogenesis.

The focus of the current study was to determine whether specific karyotypic changes are induced by the presence or absence of chronic arsenite exposure and hsa-miR-186 overexpression. We hypothesized that hsa-miR-186 overexpression contributes to malignant transformation of HaCaT cells by induction of chromosomal instability. Furthermore, we proposed that chromosomal instability and transformation will be accelerated in HaCaT cells overexpressing hsa-miR-186 with exposure to arsenite compared to HaCaT cells exposed to arsenite without hsa-miR-186 overexpression. To test this hypothesis, we had two main goals. First, we wanted to compare timing of transformation of HaCaT cells overexpressing hsa-miR-186 and exposed to arsenite with cells exposed to arsenite without hsa-miR-186 overexpression. Second, we wanted to characterize the chromosomal instability in HaCaT cells overexpressing hsa-miR-186 with or without chronic arsenite exposure.

During tumorigenesis, epithelial-to-mesenchymal transition (EMT) may increase the motility and invasiveness of cancer cells. Malignant transformation is associated with signaling pathways promoting EMT (Kalluri and Weinberg 2009).Specifically, during carcinogenesis cadherin switching may occur (Kalluri and Weinberg 2009). The latter refers to a switch from expression of E-cadherin (an epithelial marker) to expression of N-cadherin (a mesenchymal marker) [330]. One mechanism that most likely regulates cadherin switching is transcriptional repression of E-cadherin by transcriptional repressor proteins (e.g. snail, slug) (Kalluri and Weinberg 2009). Also, β-catenin is a transcription factor in the WNT signaling pathway and is involved in the regulation of cell adhesion (Polette, Mestdagt et al. 2007). β-catenin is typically more abundant in epithelial-like cells and it is reduced in mesenchymal-like cells, such as cancer cells (Polette, Mestdagt et al. 2007). Claudin 1 is a membrane protein involved in the formation of tight junctions, mainly found in epithelial cells (Kalluri and Weinberg 2009). Claudin 1 regulates transepithelial transport and plays a critical role for cell growth and differentiation (Kalluri and Weinberg 2009). Claudin 1 levels are frequently reduced in many cancers though transcriptional repression by snail or slug, during EMT (Kalluri and Weinberg 2009). Also, zona-occludens 1 (ZO-1) is a tight junction protein that is usually located at cell-cell adhesion membrane complexes in normal epithelial cells (Scanlon, Van Tubergen et al. 2013). The cytoplasmic/nuclear delocalization of ZO-1 from the tight junctions is a common process in EMT and associated with tumor invasion

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(Polette, Mestdagt et al. 2007). To elucidate the association of hsa-miR-186 overexpression with the progression to arsenic-induced skin cancer, we determined the expression of epithelial and mesenchymal markers in HaCaT cells that overexpress hsa-miR-186 exposed to 0 or 100 nM sodium arsenite, and in empty vector transfected cells exposed to 0 or 100 nM sodium arsenite.

After 29 weeks of culturing under selective arsenite pressure, we observed that only slug showed significant change in expression. Specifically, slug expression was higher in hsa-miR-186 overexpressing HaCaT cells than in vector control transfected HaCaT cells, and that elevated expression in hsa-miR-186 overexpressing HaCaT cells was reduced by chronic arsenite exposure (Figure 4). Analysis using bioinformatic tools (miRbase, TargetScan) does not predict slug as a hsa-miR-186 target, thus, hsa-miR-186 might induce slug overexpression indirectly by suppressing an inducing factor.

Despite the minimal evidence of EMT, clones of HaCaT cells overexpressing hsa-miR-186 and exposed to 100 nM sodium arsenite for 29 weeks showed strong evidence of anchorage independent growth, a clear sign of malignant transformation. Clones of cells overexpressing hsa-miR-186 and not exposed to arsenite, and clones of empty vector transfected cells regardless of arsenite exposure all showed baseline levels of colony formation in soft agar. Thus, the combination of hsa-miR-186 overexpression and chronic arsenite exposure transformed cells sooner than either hsa-miR-186 overexpression or chronic arsenite exposure alone.

Wu et al, 2018 showed that chronic arsenite exposure for 4 or 8 weeks increases aneuploidy in hsa-miR-186 overexpressing cells but not in the absence of hsa-miR-186 (Wu, Ferragut Cardoso et al. 2019). This increase in aneuploidy includes induction of double minute chromosomes, suggesting that gene amplification is occurring (Burkard and Weaver 2017). We then performed a more detailed karyotypic analysis to screen for specific chromosomal rearrangements due to arsenic exposure, hsa-miR-186 overexpression or the combination, that could drive the transformation process of HaCaT cells overexpressing hsa-miR-186 exposed to arsenite. We observed a trend towards increased structural chromosomal instability after only nine weeks of culture when there is hsa-miR-186 overexpression and concurrent arsenite exposure. Similar observations in another human keratinocyte cell line (Ker-CT) were made; increases in tetraploidy and aneuploidy occurred in hsa-miR-186 overexpressing cells after only 4 weeks exposure to 100 nM arsenite (Ferragut Cardoso, Nail et al. 2023). Aneuploidy is a well established hallmark of malignant cells. Indeed, these cultures of cells overexpressing hsa-miR-186 and exposed to arsenite are those that formed anchorage independent colonies (Figure 4).

We also observed that there is a trend towards increased number of double minutes in arsenite-exposed hsa-miR-186 overexpressing cells consistent with observation with these cells at earlier time points (Wu, Ferragut Cardoso et al. 2019). Double minute chromosomes often lack regulatory elements, and they frequently harbor amplified oncogenes (Turner, Deshpande et al. 2017). Thus, these levels of double minute chromosomes might indicate amplification of an oncogene involved in carcinogenic transformation.

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Detailed karyotypic analysis showed which structural abnormalities frequently occurred. The hsa-miR-186 overexpressing clones exposed to arsenite exhibited some structural chromosomal abnormalities more frequently compared to unexposed vector control transfected cells (Figure 2). These abnormalities included deletion of chromosomal material in chromosome 1, extra chromosomal material of unknown origin in chromosomes 11p and 20q and extra marker chromosomes (Figure 2). Analysis of the genes located in those regions could elucidate gene dosage effects that could be implicated in transformation as well as unravel pathways that lead to the acquisition of tumorigenic phenotype.

The gains of unidentified chromosomal material in chromosomes 11p and 20q, as well as the deletion of chromosomal material in chromosome 1, could be potential sites of chromosomal translocations and gene fusion events. Gene fusions represent an important class of somatic alterations in cancers, and it is estimated that they drive the development of approximately 17% of cancer cases (Sansregret, Vanhaesebroeck et al. 2018). The evidence of chromosomal translocations suggests a possible formation of gene fusions, which could contribute to carcinogenesis. Overall, our karyotypic observations suggest that hsa-miR-186 overexpression causes chromosomal instability which is further exacerbated by arsenite. This study showed that HaCaT cells overexpressing hsa-miR-186 and exposed to arsenite had increased chromosomal instability that has been associated with skin cancer progression, and that these cells also showed evidence of transformation *in vitro*.

Carcinogenesis is a multistage process, in which cancer cells evolve in response to multiple hits. Overexpression of hsa-miR-186 could be a second hit that exacerbates the effect of the first hit, which is the arsenite exposure. Not all cells within each clone are going to transform simultaneously because the transformation process is stochastic (Foo, Leder et al. 2011). The latter explains the karyotypic variability that we observed (Figures 1 and 2). Cells that will acquire beneficial phenotypic traits, such as sustained proliferative signaling or resistance to death, will clonally expand and outcompete less fit neighboring cells (Giam and Rancati 2015).

The novelty of this study is the emerging link of miRNA dysregulation and chromosomal instability, during clonal variation, as a suggested mechanism of arsenic-induced carcinogenesis. The presence of gene fusion events as an outcome of transformation stochasticity, is a novel concept in the field of arsenic carcinogenesis. The clonal variability is a fundamental principle in cancer biology and identifying and quantifying these gene fusion events could unravel the molecular pathways which mediate arsenic carcinogenesis and be potential targets for attenuating and treating arsenic-induced malignant transformation. We observed that hsa-miR-186 was overexpressed in arsenic induced squamous cell carcinomas relative to premalignant hyperkeratoses (States 2015). Recently, we showed that hsa-miR-186 overexpression induced tetraploidy and aneuploidy and targeted regulators of mitosis in another human keratinocyte cell line (Ferragut Cardoso, Nail et al. 2023). Thus, the involvement of hsa-miR-186 in the mechanisms of arsenic-induced malignant transformation and it overexpression in malignant vs pre-malignant lesions, suggests that hsa-miR-186 could serve as a potential biomarker for the progression from premalignant lesions to metastatic carcinoma.

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Supplementary Material

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References

Al-Eryani L, Jenkins SF, States VA, Pan J, Malone JC, Rai SN, Galandiuk S, Giri AK and States JC (2018). “miRNA expression profiles of premalignant and malignant arsenic-induced skin lesions.” PLoS One 13(8): e0202579. [PubMed: 30114287]

Al-Eryani L, Waigel S, Tyagi A, Peremarti J, Jenkins SF, Damodaran C and States JC (2018). “Differentially Expressed mRNA Targets of Differentially Expressed miRNAs Predict Changes in the TP53 Axis and Carcinogenesis-Related Pathways in Human Keratinocytes Chronically Exposed to Arsenic.” Toxicol Sci 162(2): 645–654. [PubMed: 29319823]

Borowicz S, Van Scoyk M, Avasarala S, Karuppusamy Rathinam MK, Tauler J, Bikkavilli RK and Winn RA (2014). “The soft agar colony formation assay.” J Vis Exp(92): e51998.

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A and Fusenig NE (1988). “Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line.” J Cell Biol 106(3): 761–771. [PubMed: 2450098]

Burkard ME and Weaver BA (2017). “Tuning Chromosomal Instability to Optimize Tumor Fitness.” Cancer Discov 7(2): 134–136. [PubMed: 28167614]

Cardoso APF, Al-Eryani L and States JC (2018). “Arsenic-Induced Carcinogenesis: The Impact of miRNA Dysregulation.” Toxicol Sci 165(2): 284–290. [PubMed: 29846715]

Chen Y, Graziano JH, Parvez F, Liu M, Slavkovich V, Kalra T, Argos M, Islam T, Ahmed A, Rakibuz-Zaman M, Hasan R, Sarwar G, Levy D, van Geen A and Ahsan H (2011). “Arsenic exposure from drinking water and mortality from cardiovascular disease in Bangladesh: prospective cohort study.” Bmj 342: d2431. [PubMed: 21546419]

Council, N. R. (2001). Arsenic in Drinking Water: 2001 Update. Washington, DC, The National Academies Press.

Ferragut Cardoso AP, Banerjee M, Al-Eryani L, Sayed M, Wilkey DW, Merchant ML, Park JW and States JC (2022). “Temporal Modulation of Differential Alternative Splicing in HaCaT Human Keratinocyte Cell Line Chronically Exposed to Arsenic for up to 28 Wk.” Environ Health Perspect 130(1): 17011. [PubMed: 35072517]

Ferragut Cardoso AP, Nail AN, Banerjee M, Wise SS and States JC (2023). “miR-186 induces tetraploidy in arsenic exposed human keratinocytes.” Ecotoxicol Environ Saf 256: 114823. [PubMed: 36989553]

Foo J, Leder K and Michor F (2011). “Stochastic dynamics of cancer initiation.” Phys Biol 8(1): 015002. [PubMed: 21301064]

Giam M and Rancati G (2015). “Aneuploidy and chromosomal instability in cancer: a jackpot to chaos.” Cell Div 10: 3. [PubMed: 26015801]

Hanlon DP and Ferm VH (1986). “Concentration and chemical status of arsenic in the blood of pregnant hamsters during critical embryogenesis. 1. Subchronic exposure to arsenate utilizing constant rate administration.” Environ Res 40(2): 372–379. [PubMed: 3732208]

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Hunt KM, Srivastava RK, Elmets CA and Athar M (2014). "The mechanistic basis of arsenicosis: pathogenesis of skin cancer." Cancer Lett 354(2): 211–219. [PubMed: 25173797]

Kalluri R and Weinberg RA (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest 119(6): 1420–1428. [PubMed: 19487818]

Kapaj S, Peterson H, Liber K and Bhattacharya P (2006). "Human health effects from chronic arsenic poisoning--a review." J Environ Sci Health A Tox Hazard Subst Environ Eng 41(10): 2399–2428. [PubMed: 17018421]

Li H, Yin C, Zhang B, Sun Y, Shi L, Liu N, Liang S, Lu S, Liu Y, Zhang J, Li F, Li W, Liu F, Sun L and Qi Y (2013). "PTTG1 promotes migration and invasion of human non-small cell lung cancer cells and is modulated by miR-186." Carcinogenesis 34(9): 2145–2155. [PubMed: 23671127]

Martinez VD, Vucic EA, Becker-Santos DD, Gil L and Lam WL (2011). "Arsenic exposure and the induction of human cancers." J Toxicol 2011: 431287. [PubMed: 22174709]

Ochi T, Kita K, Suzuki T, Rumpler A, Goessler W and Francesconi KA (2008). "Cytotoxic, genotoxic and cell-cycle disruptive effects of thio-dimethylarsinate in cultured human cells and the role of glutathione." Toxicol Appl Pharmacol 228(1): 59–67. [PubMed: 18207211]

Paranjape T, Slack FJ and Weidhaas JB (2009). "MicroRNAs: tools for cancer diagnostics." Gut 58(11): 1546–1554. [PubMed: 19834118]

Pi J, Diwan BA, Sun Y, Liu J, Qu W, He Y, Styblo M and Waalkes MP (2008). "Arsenic-induced malignant transformation of human keratinocytes: involvement of Nrf2." Free Radic Biol Med 45(5): 651–658. [PubMed: 18572023]

Polette M, Mestdagt M, Bindels S, Nawrocki-Raby B, Hunziker W, Foidart JM, Birembaut P and Gilles C (2007). "Beta-catenin and ZO-1: shuttle molecules involved in tumor invasion-associated epithelial-mesenchymal transition processes." Cells Tissues Organs 185(1–3): 61–65. [PubMed: 17587809]

Sansregret L, Vanhaesebroeck B and Swanton C (2018). "Determinants and clinical implications of chromosomal instability in cancer." Nat Rev Clin Oncol 15(3): 139–150. [PubMed: 29297505]

Sarma N (2016). Skin Manifestations of Chronic Arsenicosis. Arsenic: Exposure Sources, Health Risks and Mechanisms of Toxicity. States JC. Hoboken, NJ, John Wiley & Sons, Inc.: 127–136.

Scanlon CS, Van Tubergen EA, Inglehart RC and D'Silva NJ (2013). "Biomarkers of epithelial-mesenchymal transition in squamous cell carcinoma." J Dent Res 92(2): 114–121. [PubMed: 23128109]

Smith AH, Lingas EO and Rahman M (2000). "Contamination of drinking-water by arsenic in Bangladesh: a public health emergency." Bull World Health Organ 78(9): 1093–1103. [PubMed: 11019458]

Solomon MJ and Burton JL (2008). "Securin' M-phase entry." Nat Cell Biol 10(4): 381–383. [PubMed: 18379598]

States JC (2015). "Disruption of Mitotic Progression by Arsenic." Biol Trace Elem Res 166(1): 34–40. [PubMed: 25796515]

Su BB, Zhou SW, Gan CB and Zhang XN (2018). "MiR-186 inhibits cell proliferation and invasion in human cutaneous malignant melanoma." J Cancer Res Ther 14(Supplement): S60–s64. [PubMed: 29578151]

Sun Y, Pi J, Wang X, Tokar EJ, Liu J and Waalkes MP (2009). "Aberrant cytokeratin expression during arsenic-induced acquired malignant phenotype in human HaCaT keratinocytes consistent with epidermal carcinogenesis." Toxicology 262(2): 162–170. [PubMed: 19524636]

Taylor BF, McNeely SC, Miller HL and States JC (2008). "Arsenite-induced mitotic death involves stress response and is independent of tubulin polymerization." Toxicol Appl Pharmacol 230(2): 235–246. [PubMed: 18485433]

Turner KM, Deshpande V, Beyter D, Koga T, Rusert J, Lee C, Li B, Arden K, Ren B, Nathanson DA, Kornblum HI, Taylor MD, Kaushal S, Cavenee WK, Wechsler-Reya R, Furnari FB, Vandenberg SR, Rao PN, Wahl GM, Bafna V and Mischel PS (2017). "Extrachromosomal oncogene amplification drives tumour evolution and genetic heterogeneity." Nature 543(7643): 122–125. [PubMed: 28178237]

Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, Dalamagas T and Hatzigeorgiou AG (2015). "DIANA-miRPath v3.0: deciphering microRNA

function with experimental support." Nucleic Acids Res 43(W1): W460–466. [PubMed: 25977294]

Wu J, Ferragut Cardoso AP, States VAR, Al-Eryani L, Doll M, Wise SS, Rai SN and States JC (2019). "Overexpression of hsa-miR-186 induces chromosomal instability in arsenic-exposed human keratinocytes." Toxicol Appl Pharmacol 378: 114614. [PubMed: 31176655]

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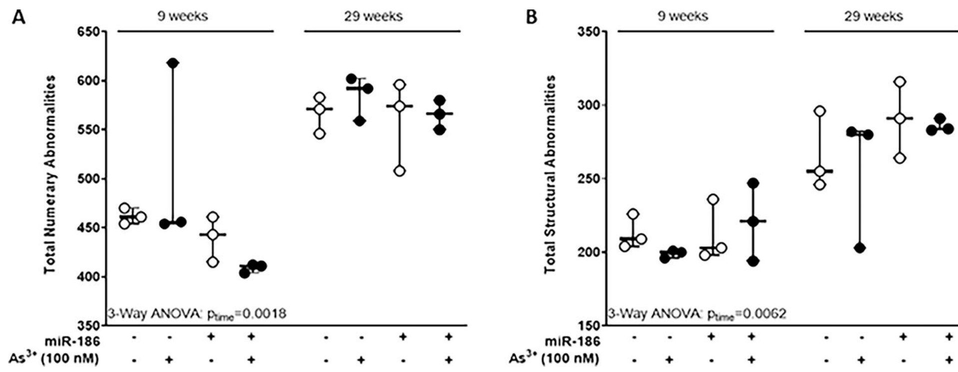

Figure 1.

Total supernumerary and structural chromosomal abnormalities in vector control and hsa-miR-186 overexpressing HaCaT cells exposed to 0 or 100 nM $NaAsO_2$ at 9 and 29 weeks. Different data points represent different clones. A. Quantification of total supernumerary chromosomal abnormalities. B. Quantification of structural chromosomal abnormalities. There is a statistically significant effect of time (p=0.0018) for the acquisition of both structural chromosomal abnormalities and supernumerary chromosomes.

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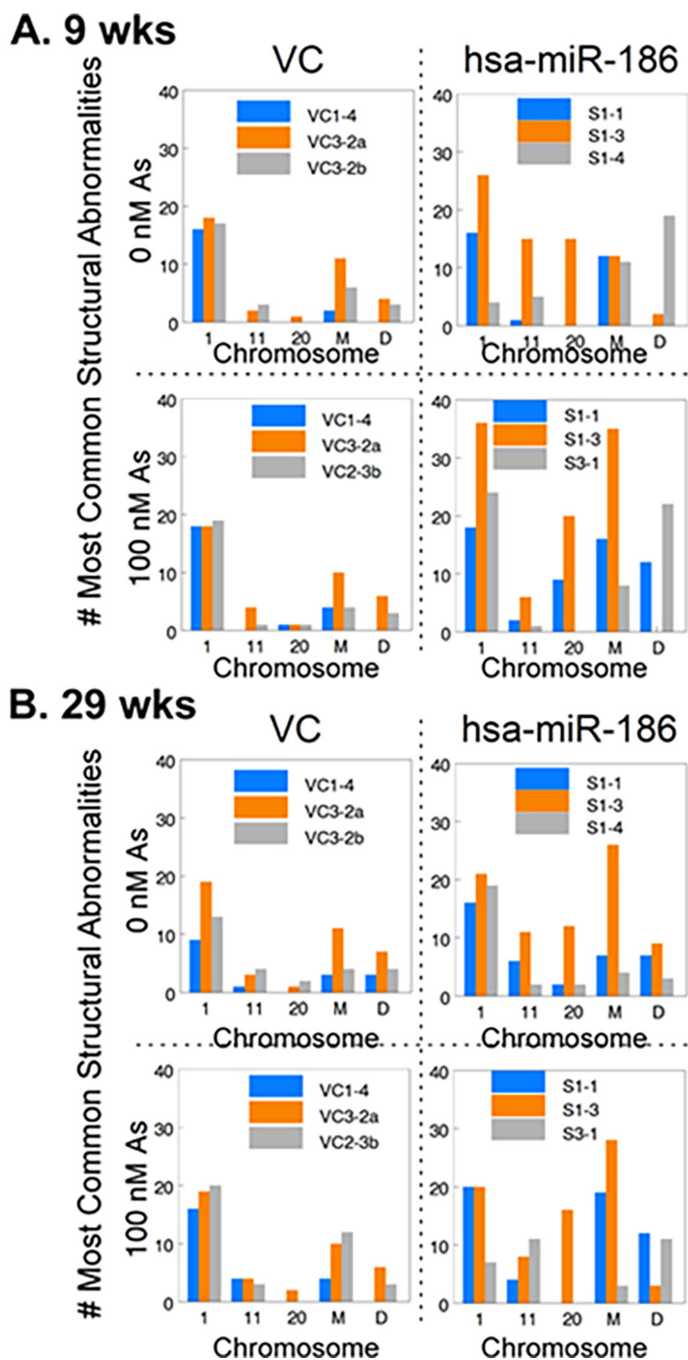

Figure 2.

Most common structural chromosomal abnormalities in vector control and hsa-miR-186 overexpressing HaCaT cells exposed to 0 or 100 nM $NaAsO_2$ at 9 and 29 weeks. Number of the most common structural chromosomal abnormalities for the three vector control clones (VC1–4, VC3–2a, VC3–2b) exposed to 0 or 100 nM $NaAsO_2$and for the three hsa-miR-186 overexpressing clones (S1-1, S1-3, S3-1) exposed to 0 or 100 nM $NaAsO_2$. 1 indicates structural chromosomal instability (deletion) in chromosome 1p, 11 indicates structural chromosomal instability (addition of uknown chromosomal material) in chromosome 11p,

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20 indicates structural chromosomal instability (addition of uknown chromosomal material) in chromosome 20q, M indicates extra marker chromosomes, D indicates double minute chromosomes. A. Quantification of the above structural chromosomal abnormalities at 9 weeks. B. Quantification of the above structural chromosomal abnormalities at 29 weeks

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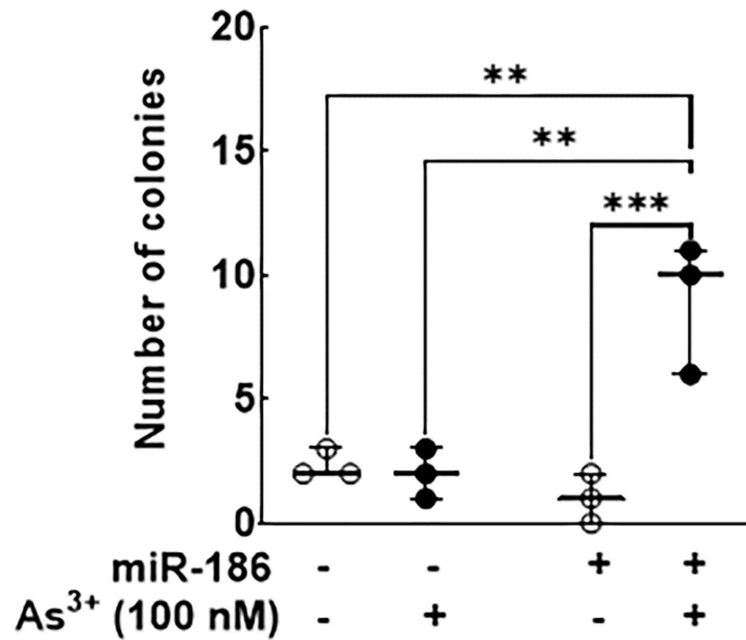

Figure 3.

Number of colonies in vector control and hsa-miR-186 overexpressing HaCaT cells exposed to 0 or 100 nM $NaAsO_2$ 29 weeks. Different data points represent different clones. There is a statistically significant colony formation capability of arsenic exposed hsa-miR-186 overexpressing HaCaT cells compared to all other cells.

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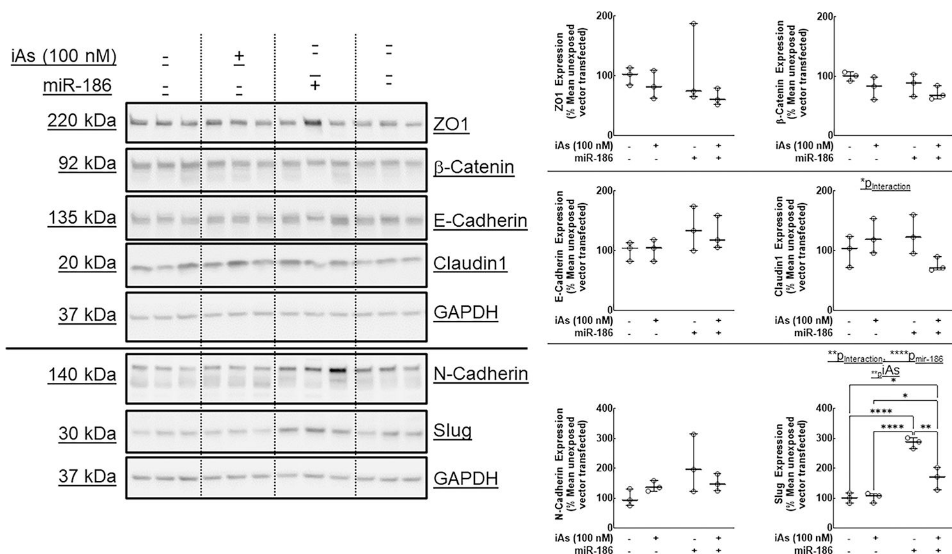

Figure 4.

Western blots for epithelial-mesenchymal transition markers in vector control and hsa-miR-186 overexpressing HaCaT cells exposed to 0 or 100 nM $NaAsO_2$ at 29 weeks. Different data points represent different clones. Quantification of marker expression relative to GAPDH by densitometric analysis. Slug expression is significantly increased in HaCaT cells overexpressing hsa-miR-186 regardless of arsenite exposure compared to vector control cells. Data are represented as the % of the mean control expression ± SD. Data were analyzed with two-way ANOVA and Tukey's multiple comparison post hoc test. *p<0.05; **p<0.01; ****p<0.0001