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Arsenic-exposed Keratinocytes Exhibit Differential microRNAs Expression Profile; Potential Implication of miR-21, miR-200a and miR-141 in Melanoma Pathway

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Abstract

Long-term exposure to arsenic has been linked to cancer in different organs and tissues, including skin. Here, non-malignant human keratinocytes (HaCaT) were exposed to arsenic and its effects on microRNAs (miRNAs; miR) expression were analyzed *via* miRCURY LNA array analyses. A total of 30 miRNAs were found differentially expressed in arsenic-treated cells, as compared to untreated controls. Among the up-regulated miRNAs, miR-21, miR-200a and miR-141, are well known to be involved in carcinogenesis. Additional findings confirmed that those three miRNAs were indeed up-regulated in arsenic-stimulated keratinocytes as demonstrated by quantitative PCR assay. Furthermore, bioinformatics analysis of both potential cancer-related pathways and targeted genes affected by miR-21, miR-200a and/or miR-141 was performed. Results revealed that miR-21, miR-200a and miR-141 are implicated in skin carcinogenesis related with melanoma development. Conclusively, our results indicate that arsenic-treated keratinocytes exhibited alteration in the miRNAs expression profile and that miR-21, miR-200a and miR-141 could be promising early biomarkers of the epithelial phenotype of cancer cells and they could be potential novel targets for melanoma therapeutic interventions.

Keywords

Arsenite; cancer; carcinogenesis; miR-21; miR-200a; miR-141; miRNA-based cancer therapy; skin cancer

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

INTRODUCTION

Exposure to the metalloid arsenic around the world is a complex global environmental problem. Humans are frequently in contact to arsenic due that is a natural environmental pollutant of water, air, soil and food [1]. Arsenic is an element that occurs in the minerals surrounding aquatic underground reservoirs and it leaches to deposits that have been stored for prolonged time periods [2–4] resulting in extensive human exposure throughout the world. Several epidemiological studies have correlated the arsenic exposure with increases in the incidence of skin, liver, bladder, and lung cancer [5, 6]. This problem has been exacerbated by an increment in the use of arsenic contaminated water, especially in developing countries [7]. Moreover, melanoma is a skin cancer type that has an unfavorable prognosis and in developed countries is the main cause of death from skin cancers, with extensive influence of environmental factors to its etiology [8]. As one of the deadliest human cancers, malignant melanoma frequently conducts to death within a year, when it converts metastatic [9]. As a result, the Environmental Protection Agency in the United States, the European Union and the World Health Organization have adopted a 10 ppb limit for arsenic in drinking water and there are plans to reduce these limits to lower levels [10, 11]. For these reasons, there is a need to improve our knowledge of the molecular effects following arsenic exposure and its association with skin cancer, to reveal novel approaches for the design of melanoma therapeutic strategies.

MiRNAs are a big family of short single-stranded (~22 nucleotides) non-coding RNA molecules endogenously produced that are transcribed in animals, plants and some viruses, suppressing target gene translation by binding to their mRNA [12]. Also, miRNAs function as versatile gene expression regulators in virtually all cellular pathways in higher eukaryotes, including proliferation, apoptosis, cell differentiation, cell cycle, embryonic development and cellular disease [13, 14]. Additionally, miRNAs expression is altered in cancer emergence and metastatic occurrences, therefore having a pivotal role in health and disease [13]. The miRNAs family, grouping 1881 miRNAs sequences from *Homo sapiens* (hsa), is known to regulate the expression of more than 60% of human protein-coding genes [15–17]. Thus, miRNAs revealed an additional mechanism by which genes are regulated and added yet another dimension of complexity to the regulation of biological systems [18, 19]. The expression of miRNAs is affected by several toxicants and cellular stressors [20] and they can be used as novel biomarkers of toxicological insult [21].

Experimental manipulation has shown an important role of miRNAs as regulators in carcinogenesis and possibly in anti-cancer therapy [22]. For example, a recent study demonstrated that disturbing the expression of a single miRNA (miR-21) is sufficient to induce cancer in a mouse model [23]. Another experiment showed that restoring the expression of miR-26a in mice resulted in the significant size reduction of liver tumors without noticeable toxicity to healthy tissues [24]. Also, studies utilizing cutaneous squamous cell carcinoma (CSCC) derived from epidermal keratinocyte indicates that miR-31 controls cancer-related phenotypes [25]. Moreover, it was revealed that miR-20a is involved in the tumor inhibition of CSCC [26]. Due to the role of miRNAs in cancer, it is reasonable to hypothesize that the functions of these small RNA molecules acting as gene regulators are disturbed by chemical carcinogens, such as arsenic. In this context, the

investigation of miRNAs expression during arsenic exposure would open novel epigenetic perspectives to understand the toxicological effects derived by the presence of this cytotoxic metalloid in the environment, as well as in drinking water. It was proposed that more research is required to clarify the molecular mechanisms leading arsenic-driven carcinogenesis [27] and this study is a contribution to that type of endeavors, linking the miRNAs expression with skin carcinogenesis.

The non-tumorigenic human keratinocyte HaCaT cell line, at its full differentiation capacity [28], provides an excellent model to understand the effects of arsenic in the skin [29]. This cell line can be malignantly transformed after continuous exposure to low levels of sodium arsenite [30–32]. In the present study, HaCaT cells were used to investigate the effects of *in-vitro* arsenic exposure on miRNAs levels. We hypothesized that arsenite would disturb miRNAs expression in the HaCaT cells in connection with its other well-known genotoxic effects. The expression of miRNAs was investigated by using a specific Locked Nucleic Acid (LNA) miRNA array. Our data demonstrated that human epithelial HaCaT cells differentially expressed miRNAs after low concentration sodium arsenite exposure and provided information about potential epigenetic markers of arsenic-driven skin carcinogenesis.

MATERIALS AND METHODS

Cell Culture

The human keratinocyte HaCaT cell line (Cell Lines Service, Eppelheim, Germany) are spontaneously transformed to non-malignant keratinocytes that have been established from human male-derived skin cells [28, 33]. HaCaT cells exhibit a doubling time of approximately 24 hours [28]. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Consistently, cell cultures were maintained in a humidified atmosphere at 37 °C and 5% CO₂. Culture media was replaced with fresh growth media every 2–3 days. To ensure high cell viability, cells were processed as described earlier [34].

Arsenite Treatment

Aqueous solutions of sodium arsenite (NaAsO₂, Sigma Aldrich, St. Louis, MO; hereafter referred as arsenite) were prepared as concentrated stocks of 500 µM with sterile PBS (1000×), aliquoted and stored at –20 °C. HaCaT cells were seeded in eight flasks and grown to 80–90% confluence without arsenite. On the first day of exposure, cells from four flasks (control samples) were separately harvested by the use of 0.25% trypsin solution (Invitrogen, Carlsbad, CA), and stored in RNeasy lysis reagent (Qiagen, Austin, TX) at –80 °C. The cells in the remaining four experimental flasks were then treated with medium containing 500 nM arsenite for a period of four weeks. During this period of time, at approximately 80–90% confluence level (~6–7 days), the cells were harvested with 0.25% Trypsin solution and a fraction (~150 000 cells) was seeded again to continue the arsenite exposure. As a consequence of the high dilution factor of the stock arsenite solution (1:1000), the final concentration of vehicle/solvent in the cell culture media was very low, 1

μl of PBS per 1 ml of growth media and therefore the vehicle control was omitted. At the end of this period, the cells in each of the four arsenite treated flasks were independently harvested and stored in RNAlater RNA stabilization solution at -80 °C. Four untreated control samples and four arsenite-treated samples were collected, each one representing a biological replicate.

Microarray Analysis

One control sample and one experimental sample were sent to the company Exiqon Inc. (Woburn, MA) in order to perform miRNA microarray hybridization and analysis. The miRCURY™ RNA Isolation Kit and the miRCURY™ LNA Array power labeling kit (Exiqon) were used to extract total RNA and to label the samples. The samples were then scanned using the Agilent G2565BA Microarray Scanner System (Santa Clara, CA) The ImaGene 8.0 software was used for image analysis (BioDiscovery, Inc., Hawthorne, CA). Normalization was performed with the global Lowess regression algorithm. The miRCURY™ LNA Array contains 1,890 capture probes that cover 1,395 human miRNAs (Exiqon Inc.).

MiRNAs Isolation and cDNA Preparation

In order to validate the microarray data, a miRNA purification kit (Biosynthesis, Lewisville TX) was used to extract miRNAs from the remaining biological replicates of the untreated control (N=3) and arsenite-treated (N=3) samples. Subsequently, cDNA was synthesized for each sample using the miRNA First-Strand synthesis kit following manufacturer's instructions (Clontech, Mountain View, CA). Briefly, 3.75 μl of miRNA sample were combined with 5 μl of mRQ Buffer (2×) and 1.25 μl of mRQ enzyme. The samples were incubated for 1 hour at 37 °C in a thermocycler and then, diluted with 90 μl of ddH₂O.

Quantitative PCR (qPCR) Analysis

MiRNA primers, used to amplify their entire sequence, were designed for five selected miRNAs (Integrated DNA technologies Inc, Coralville, IA). The miRNAs expression was quantified using the SYBR qRT-PCR kit (Clontech) and the cDNA from each of the three biological replicates of arsenite-treated (N=3) and control samples (N=3). For each biological replicate the quantitative PCR (qPCR; real time PCR) assay was run in duplicates and miR-191 was used for normalization purposes, as recommended in the literature [35]. For data analysis, the fold change values were obtained by the delta-delta Ct (cycle threshold) method [36].

Potential Signaling Pathways and Target Genes

The DIANA-mirPath computational algorithm [37] was used to identify potential molecular pathways related to the three miRNAs whose differential expression was significantly overexpressed following qPCR analysis (miR-21, miR-200a and miR-141) as well as common gene targets of these miRNAs.

Statistical Analyses

Two-tailed paired Student's *t*-tests were accomplished to find the statistical significance of the differences between the mean values of experimental and control samples; arsenite-treated (N=3) vs. untreated (N=3) cells. Statistical significance was deemed for *p* values < 0.05.

RESULTS

Arsenic Exposure Caused Differential Expression of miRNAs

The microarray-based analysis showed thirty differentially expressed miRNAs in the miRNA samples from arsenite-treated HaCaT cells, as compared to the untreated controls (Table 1). Based on the fold change difference (cutoff expression value was of >1.5), twenty one of them were up-regulated and nine were down-regulated. The highest fold change difference was for miR-22 (2.43 fold) and the lowest for miR-PLUS-A1087 (0.33 fold). Some of the differentially expressed miRNAs are linked to carcinogenesis: miR-22 [38], miR-21, miR-34b [39], miR-141, miR-200a [40], miR-27b and miR-23b [41]. Six of the nine down-regulated miRNAs are denoted as miRPLUS. These six sequences are present in the miRCURY Exiqon array and are licensed human sequences that were not yet annotated in the miRBase at the time of the hybridization. These miRNAs were discovered by Exiqon which provided novel information about miRNAs that were not available in the literature.

Quantitative PCR Analysis

Five miRNAs were selected for further analysis *via* qPCR to corroborate their expression levels obtained by microarray-based assay. Four of them were selected, based on their notorious differential expression and their relevance in carcinogenesis, and these were miR-21, miR-34b, miR-141 and miR-200a. The other miRNA, miR-PLUS-A1087, was selected for validation because it was the miRNA with greatest expression change (Table 1). The results acquired by both qPCR and microarray assays for those miRNAs are presented in (Table 2). Three of them, miR-21, miR-200a and miR-141, were found to be up-regulated based on both the microarray and qPCR analyses techniques, and their *p* value of the qPCR fold change for all of them was consistently statistically significant ($p < 0.05$), as compared with untreated controls (Table 2). The miRPLUS-A1087 was downregulated in the array and in qPCR but the qPCR fold change was marginally significant for this miRNA (Table 2). MiR-34b was down-regulated based on the array and up-regulated based on qPCR, although the fold change was not statistically significant (Table 2).

Potential Signaling Pathways of Selected Target Genes

To establish the importance of miR-21, miR-200a and miR-141 expression on arsenite-treated HaCaT cells and to investigate their involvement in the cellular metabolism context, the DIANA-mirPath computational database was utilized for *in silico* analysis. This database is designed to identify molecular pathways that are affected by single or multiple miRNAs occurrences. The DIANA-mirPath analysis revealed that at least one or more of the three selected miRNAs targeted genes involved in 16 pathways relevant to carcinogenesis (Table 3). The KEGG mitogen-activated protein kinase (MAPK) signaling pathway was the most

pronounced, comprising potential alteration of 27 target genes within its metabolic pathway (map04010). Additionally, the KEGG melanoma pathway (map05218), which is the most relevant pathway implicated in the skin carcinogenesis, was found to be affected by those three miRNAs and some of their potential targets genes are depicted in (Fig. 1). Other pathways appeared to be also potentially altered, including the Jak-STAT and several cancer related pathways (prostate, pancreatic and lung).

The DIANA-miRPath data base was also used to find an intersection of potential target genes of miR-21, miR-200a and miR-141. We found common potential targets affected by all three miRNAs simultaneously (Fig. 1 and Table 4). Genes involved in the TGF-beta signaling pathway followed by actin regulation and cytokine pathways were the most commonly regulated by the three selected miRNAs. In addition, a group of target genes was also identified, participating in more than one biochemical pathway, including cyclin-dependent kinase 6, bone morphogenetic protein receptor type II, activin receptor type 2A and phosphatidylinositol-3-phosphate/5-kinase genes (Fig. 1 and Table 4).

DISCUSSION

The aim of this study was to investigate differential expression of miRNAs in human HaCaT keratinocytes after low-dose arsenic exposure. Previous reports revealed that long-term (20 or 28 weeks) low-dose exposure to sodium arsenite (0.1 or 0.5 or 1 μ M) transformed non-malignant human cells, HaCaT keratinocytes, into a cancer cell phenotype or tumorigenic cells [30–32]. Thus, it was decided to expose the HaCaT cells to 500 nM of arsenite and study their miRNAs expression; an average arsenite concentration previously reported [30–32]. Five out of the thirty miRNAs identified to be altered by microarray analysis were selected for further validation *via* qPCR; on the basis of significant fold change expression (Table 2). Three out of those five, miR-21, miR-200a and miR-141, were found to be up-regulated by both microarray and qPCR assays (Table 2).

In this report, the simultaneous up-regulation of miR-21, miR-200a and miR-141 provided an interesting point of intersection to understand convergent mechanisms of arsenic toxicology and to predict with more accuracy potential carcinogenetic targets of this metalloid. Moreover, our findings could be applied to additional metals- and metalloid-related toxicological disorders. Through the modulation of these three miRNAs, arsenic may affect several signaling pathways related to cell growth and proliferation as well as apoptosis regulation (Table 3). Some of the signaling pathways that are potentially regulated by miR-21, miR-200a and miR-141 include pathways with a well-known role in carcinogenesis such as the MAPK, Jak-STAT and Wnt pathways (Table 3). These pathways have also been reported in the literature to be disturbed by arsenic exposure and contain many genes in common [42–44]. For these reasons, the genes that are simultaneously targeted by these three miRNAs throughout these pathways (Table 4) constitute an interesting cluster of genetic biomarkers that deserve special attention for future research and therapy design in skin carcinogenesis. In addition, these findings highlight the complexity of miRNA-mediated regulatory networks.

MiR-21 expression is involved in several pathologies from cancer to cardiovascular disease [45]. MiR-21 is over-expressed in most human tumors [46], including early pancreatic cancer lesions, pancreatic tumors, and pancreatic cancer-derived cell lines [47], breast [48], cervix [49], lung [50], and other types of cancer (for review, see Jazbutyte *et al.* 2010 [45]). This miRNA is one of the most frequently disturbed miRNA during carcinogenesis and its induction has been related to reduction of apoptosis [51] and is the most dramatically overexpressed miRNA in both solid and hematological malignancies [50]. Moreover, miR-21 up-regulation is commonly associated with poor prognosis in cancer patients and has been suggested to be a potential useful biomarker for cancer prognosis, as well as a potential therapeutic target in various diseases [25, 45, 47]. A recent report indicated that miR-21 inhibits the tumor suppressive activity of FBXO11 to promote tumorigenesis in melanoma, glioblastoma and prostate cancer [52]. For instance, it was found that by using an antisense suppressor of miR-21, this treatment significantly sensitized the K562 leukemic cells to arsenic trioxide by promoting apoptosis [53]. Thus, the regulation of miRNAs expression in combination with well-known anti-cancer drugs remains as a promising strategy for miRNA-based anticancer therapy.

Previous report proved that miR-141 and its targets PH domain leucine-rich-repeats protein phosphatase 1 and 2 (PHLPP1 and PHLPP2) play key roles in non-small cell lung cancer tumorigenesis [54]. MiR-141 was found to be significantly down-regulated in pancreatic cancer tissues and cell lines and also was found that miR-141 targets MAP4K4, which acts as a tumor suppressor in pancreatic cancer cells, providing a novel therapeutic option for miRNA-based pancreatic cancer therapy [55]. It was proposed that miR-141 expression is involved with the progression of bladder carcinoma and its overexpression is independently associated with favorable prognosis of patients affected with bladder cancer [25]. Also, MiR-141 conferred resistance to cisplatin-induced apoptosis by targeting YAP1 in human esophageal squamous cell carcinoma [56]. In contrast, miR-141 functions as a tumor suppressor in hepatocellular carcinoma (HCC) through the inhibition of HNF-3 β expression, and suppressed proliferation and invasion by promoting the apoptosis of HCC cells [57]. Also, miR-141 inhibited tumor growth and metastasis by directly targeting transcriptional co-activator with PDZ-binding motif (TAZ) in gastric cancer [58].

It was suggested that the miR-200 family determines the epithelial-to-mesenchymal transition and cancer progression phenotype [59]. Furthermore, both in epithelial cancer cells and other well-differentiated cancers, miR-200 family members are highly expressed strongly controlling the expression of the E-cadherin repressors, zinc-finger E-box-binding homeobox factors 1 and 2 (ZEB1 and ZEB2) [59]. The miR-200 family is a biomarker for cells that express E-cadherin but lack expression of vimentin, determining the epithelial phenotype of cancer cells [59]. Additionally, ZEB2 is over-expressed in various advanced stages of ovarian, gastric, and pancreatic cancer [60–62]. The possible role for miR-200a in lung adenocarcinoma was investigated, finding that low levels of miR-200a were significantly associated with poorer overall survival rate [63]. Furthermore, the miRNA-200 family regulates cellular motility and controls “stemness” in several cancers [64, 65]. Conversely, breast cancer with low expression of a miR-200a was correlated with improvement of clinical prognosis [63]. MiR-200 delivered into ovarian, lung, renal and

basal-like breast cancers resulted in blocking cancer angiogenesis and suppressing metastasis [63]. Thus, miRNA-200 could be utilized also as potential therapeutic agents.

It is well documented that arsenite, or other arsenic-derived compounds, induces oxidative stress and apoptosis in several cell lines, included keratinocytes. A previous report proposed that arsenite exerts its cytotoxic effect on myoblasts by inducing apoptosis, evidenced by reactive oxygen species (ROS)-induced mitochondrial dysfunction, endoplasmic reticulum stress, and Akt inactivation signaling pathway [66]. Also, arsenite was found to induce apoptosis in lymphoblastic cells [67]. Additionally, results indicated that arsenic-induced ROS apoptosis *via* several downstream alterations including genotoxicity, signal transduction, cell proliferation and inhibition of DNA repair [1]. Moreover, as consequence of long-term arsenite exposure, mouse lymphoid A20 cells manifested premature senescence through enhance DNA damage and p130 accumulation [68]. In cultured lung epithelial cells exposed to low levels of arsenic triggered oxidative stress and also activated the expression of genes related to stress [69]. In keratinocytes, NF- κ B activity was increased by low concentrations of arsenic promoting proliferation, in contrast, the activity of NF- κ B was not increased with high arsenic concentrations and Fas/FasL-associated apoptosis was induced; suggesting that the change of NF- κ B activity is associated with arsenic-induced keratinocyte proliferation and cell death *via* the apoptosis pathway [70]. These results were confirmed for an additional study, concluding that arsenite, as well as arsenic pentoxide and arsenic iodide, was capable of inducing programmed cell death *via* DNA fragmentation in cultured HaCaT keratinocytes [71]. Moreover, it was proposed that arsenic-induced ROS formation provide evidence of apoptotic DNA fragmentation in keratinocytes, which may be implicated in the mechanism for arsenic-induced skin carcinogenicity [72]. Thus, arsenite-induced cytotoxicity involves oxidative stress, increases ROS production and DNA fragmentation, which cause apoptosis.

Arsenite-induced malignant transformation of human embryo lung fibroblast (HEL F) cells was mediated by miR-21 overexpression, which was facilitated by ROS activation of the ERK/NF- κ B pathway [73]. Additionally, it was proposed that miR-21 levels were over-expressed by the activations of ERK/NF- κ B and JNK/c-Jun, and concomitantly, miR-21 inhibited target proteins of Pdc4 and Spry1, which function as two feedback regulation loops of miR-21-Spry1-ERK/NF- κ B and miR-21-Pdc4-JNK/c-Jun in arsenite-induced transformation of HEL F cells [74]. Also, it was reported that arsenic-exposed human umbilical vein endothelial (HUVEC) cells expressed alteration in miRNAs expression profiles, including miR-21 [75]; however, no change in miR-141 and miR-220a was reported. Moreover, miR-21 was found to be involved in both carcinogenesis and angiogenesis after arsenite exposure of non-tumorigenic SV40-transformed HBE and HUVEC cells [76]. In human primary neonatal keratinocytes (NHEKs) it was found that arsenic causes cumulative disruptions to epigenetic regulation of miR-34a expression and alteration of its target histone deacetylase SIRT1 protein, by disrupting the profile of histone acetylation and DNA methylation and by suppressing SIRT1 activity [77]. In a separate report, it was demonstrated that acute exposure of HaCaT keratinocytes to arsenite, p21waf1/Cip1 was up-regulated and Notch1 was down-regulated, favoring the Notch1 contribution on arsenic-induced keratinocyte transformation and cancerogenesis [78]. In addition, it was reported that arsenite induced epigenetic silencing of let-7c *via* Ras/NF- κ B

that is involved in the acquisition of cancer stem cell-like properties and neoplastic transformation of HaCaT cells, which contribute to the tumorigenesis [79].

Investigating the expression profile of three cancer-associated miRNAs, miR-21, -141, and -221, in the blood of patients afflicted with metastatic prostate advanced cancer was found that they were significantly over-expressed as compared with non-metastatic cancer patients, where the alteration of the miR-141 was the most noticeable [80]. Also results suggested that both miR-141 and miR-200a are possible players in regulation of Th17/Treg differentiation and pathogenesis of multiple sclerosis [81]. Moreover, the up-regulation of miR-21, miR-200a and miR-141 in our profiling implicates them as important carcinogenic biomarkers associated with arsenite exposure. Among the potential targets regulated by miR-21, miR-200a and miR-14, there are components of the Wnt and the PI3K pathways (Table 3, Table 4). There is a lack of knowledge about the signaling pathways that regulate epigenetic events and how cells communicate these signals among them. However, the Wnt pathway has been proposed as an ideal candidate for regulation of epigenetics due to its role in several stages of cellular differentiation [82]. The PI3K signaling pathway is involved in the movement of exosomes between cells [83], as well as many processes [84]. The transport of miRNAs and other regulatory molecules through exosomes is a proposed mechanism for epigenetic communication between cells, which could have great relevance to cellular differentiation and carcinogenesis [85]. Our results suggest that these pathways are tentative toxicological targets of arsenic through the differential expression of miRNAs. Due to the potential implications of these effects in epigenetic signaling, they deserve more in depth analyses in the future. Hence, miRNAs as post-transcriptional regulators of gene expression are implicated in carcinogenesis, cancer diagnosis and prognosis, as well as potential targets for miRNA-based cancer therapy, alone or in combination with anticancer drugs.

The DIANA-mirPath analysis revealed that miR-21, miR-141 and miR-200a were not associated with squamous cell carcinoma, neither basal cell carcinoma; which both are non-melanoma types of skin cancer. Nevertheless, finding that the miR-21, miR-141 and miR-200a expression levels were altered in arsenite-exposed keratinocytes and that those miRNAs were also implicated with melanoma pathway is certainly interesting; being that melanoma is the most frequent, highly aggressive and deadly skin cancer type. Furthermore, the same miRNAs alteration pattern observed in arsenite-exposed HaCaT cells should be investigated by using a melanocyte cell line or a melanocyte derived cell line.

CONCLUSION

In summary, our study reveals that several miRNAs were altered in non-malignant human keratinocytes after low-concentration arsenic exposure. Also, the potential biochemical pathways that could be affected by arsenic were investigated and three of the most altered miRNAs, miR-21, miR-200a and miR-141, were found to regulate a highly complex pattern of genes and are associated to the melanoma pathway. Furthermore, these findings could be extended to characterize the role of potential miRNAs targets in the molecular toxicology networks provoked by arsenic and their inference in carcinogenesis. Additionally, increasing our understanding of up- or down-regulation of miRNAs and their involvement in cancer, as

well as their roles in multifaceted regulatory pathways, may provide foundations for developing better therapeutic strategies.

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Fig. (1). KEGG Melanoma Pathway

MiRPath showing the genes involved in the melanoma pathway that are potentially regulated by miR-21, miR-200a and miR-141. EGFR - Epidermal Growth Factor Receptor; RAS - Rat Sarcoma proteins; PKB/AKT - Protein kinase B/AKT oncogene; RB - Retinoblastoma gene; p53-cellular tumor protein; p21- cyclin-dependent kinase inhibitor; and MITF - Microphthalmia Transcription Factor.

Table 1

Differentially expressed miRNAs in HaCaT cells after exposure to arsenite.

miRNA	Fold Change	miRNA	Fold Change	miRNA	Fold Change*
hsa-miR-22 [†]	2.43	hsa-miR-30e	1.75	hsa-miR-29b	1.63
hsa-miR-21	2.22	hsa-miR-27b	1.72	hsa-miRPLUS-E1012	0.59
hsa-miR-34a	2.04	hsa-miR-23b	1.69	hsa-miRPLUS-G1246-3p	0.58
hsa-miR-205	2.00	hsa-miR-27a	1.66	hsa-miR-1285	0.57
hsa-miR-141	1.92	hsa-miR-1274a	1.66	hsa-miRPLUS-E1247	0.57
hsa-miR-1260	1.92	hsa-miR-181a	1.66	hsa-miRPLUS-F1147	0.56
hsa-miR-720	1.88	hsa-miR-1469	1.66	hsa-miRPLUS-F1231	0.56
hsa-miR-1280	1.85	hsa-miR-19a	1.63	hsa-miR-34b	0.56
hsa-miR-200a	1.81	hsa-miR-184	1.63	hsa-miR-F1017	0.49
hsa-miR-19b	1.78	hsa-miR-101	1.63	hsa-miRPLUS-A1087	0.33

[†]The first three letters indicate the organism origin of the miRNA (miR); hsa, *Homo sapiens*. The numbering of miRNA genes is the sequential order that they were entered in the database. A LNA miRNA microarray was used to detect differential expression in the experimental and control samples.

*Fold change (FC) is the ratio of expression in the treated sample as compared to the untreated control. A fold change >1 means higher expression in the arsenite treated sample; whereas a fold change <1 means lower expression in the arsenite treated sample, as compared with the untreated control cells.

Table 2

MiRNAs expression validation by qPCR.

miRNA	qPCR Untreated Cells ($2^{\Delta(-Ct)}$)	qPCR Arsenite Treated Cells ($2^{\Delta(-Ct)}$)	qPCR Fold Change	Microarray Fold Change
miR-21	$70.44 \pm 9.17^{\dagger}$	157 ± 28.54	2.22^{**}	2.22
miR-34b	0.44 ± 0.22	0.722 ± 0.54	1.64	0.56
miR-Plus-A1087	8.6 ± 4.74	1.99 ± 1.06	0.231	0.33
miR-200a	4.35 ± 1.744	9.11 ± 1.159	2.09^{*}	1.81
miR-141	10.08 ± 4.08	24.15 ± 3.93	2.39^{*}	1.92

Ct = delta () cycle threshold.

[†] Average values and their corresponding standard deviation from triplicates.Statistical significance was calculated using Student's *t*-test in the qPCR analysis;* $p < 0.05$;** $p < 0.01$.

Table 3

Cancer-related pathways affected by miR-21, miR-200a and/or miR-141.

KEGG Pathway	Pathway ID	Number of miRNA Target Genes Within Pathway
MAPK signaling	hsa04010	27
Wnt signaling	hsa04310	18
Prostate cancer	hsa05215	12
Jak-STAT	hsa04630	17
Regulation of actin cytoskeleton	hsa04810	17
Chronic myeloid leukemia	hsa05220	10
Small cell lung cancer	hsa05222	10
Pancreatic cancer	hsa05212	9
Regulation of actin cytoskeleton	hsa04810	17
Colorectal cancer	hsa05210	9
TGF-beta signaling	hsa04350	9
Cell cycle	hsa04110	10
Melanoma	hsa05218	7
Glioma	hsa05214	6
Apoptosis	hsa04210	7
p53 signaling	hsa04115	6

The three miRNAs whose fold change was statistically significant in the qPCR analysis (miR-21, miR-200a and miR-141) were used in the DIANA-mirPath database to identify potential signaling pathways regulated by them. The ones related to carcinogenesis are shown in the list. The number of genes targeted by one or more of the significant miRNAs (miR-21, 200a or miR-141) is annotated in the right column; Number of miRNA target genes within the respective pathway.

Table 4

Targeted genes regulated simultaneously by miR-21, miR-200a and miR-141 that were up-regulated after exposure of HaCaT cells to arsenic.

Targeted Genes Regulated by miR-21, miR-200a and miR-141	KEGG Pathway	KEGG Pathway ID
Activin receptor type-2A		
Bone morphogenetic receptor	TGF-beta signaling pathway	hsa04350
Homodomain transcription factor 2		
T-cell lymphoma invasion and metastasis inducing protein 1,	Regulation of actin cytoskeleton	hsa04810
Phosphatidylinositol-3-phosphate/5-kinase		
Jagged 1 protein	Notch signaling pathway	hsa04330
Activin receptor type-2A	Cytokine-cytokine receptor interaction	hsa04060
Bone morphogenetic receptor		
Phosphatidylinositol-3-phosphate/5-kinase	Inositol phosphate metabolism	hsa00562
Cyclin-dependent kinase 6	Non-small cell lung cancer	hsa05223
Cyclin-dependent kinase 6	Glioma	hsa05214
Cyclin-dependent kinase 6	p53 signaling pathway	hsa04115
Phosphatidylinositol-3-phosphate/5-kinase	Phosphatidylinositol signaling system (PI3K)	hsa04070
Cyclin-dependent kinase 6	Melanoma	hsa05218
Cyclin-dependent kinase 6	Pancreatic cancer	hsa05212
Cyclin-dependent kinase 6	Chronic myeloid leukemia	hsa05220
Cyclin-dependent kinase 6	Small cell lung cancer	hsa05222
cAMP-responsive element-binding protein 5	Prostate cancer	hsa05215
Cyclin-dependent kinase 6	Cell cycle	hsa04110
Thyroid hormone receptor beta	Neuroactive ligand-receptor interaction	hsa04080

The genes listed in the left column are common potential targets of miR-21, miR-200a and miR-141. In the center column are annotated some pathways in which those genes are involved.