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Identification of differentially expressed genes in the livers of chronically i-As-treated hamsters

Alba Hernández[a,b], Adriana Sampayo-Reyes[c], Ricard Marcos[a,b,*]

[a] Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Spain
[b] CIBER Epidemiología y Salud Pública, ISCIII, Spain
[c] Centro de Investigaciones Biomédicas del Noreste (CIBIN), Instituto Mexicano del Seguro Social (IMSS), Monterrey, Nuevo León, Mexico

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ABSTRACT

Inorganic arsenic (i-As) is a human carcinogen causing skin, lung, urinary bladder, liver and kidney tumors. Chronic exposure to this naturally occurring contaminant, mainly via drinking water, is a significant worldwide environmental health concern. To explore the molecular mechanisms of arsenic hepatic injury, a differential display polymerase chain reaction (DD-PCR) screening was undertaken to identify genes with distinct expression patterns between the liver of low i-As-exposed and control animals. Golden Syrian hamsters (5–6 weeks of age) received drinking water containing 15 mg i-As/L as sodium arsenite, or unaltered water for 18 weeks. The *in vivo* MN test was carried out, and the frequency of micronucleated reticulocytes (MN-RETs) was scored as a measure of exposure and As-related genotoxic/carcinogenic risk. A total of 68 differentially expressed bands were identified in our initial screen, 41 of which could be assigned to specific genes. Differential level of expression of a selected number of genes was verified using real-time RT-PCR with gene-specific primers. Arsenic-altered gene expression included genes related to stress response, cellular metabolism, cell cycle regulation, telomere maintenance, cell–cell communication and signal transduction. Significant differences of MN-RET were found between treated (8.70 ± 0.02 MN/1000 RETs) and control (2.5 ± 0.70 MN/1000 RETs) groups ($P < 0.001$), demonstrating both the exposure and the i-As genotoxic/carcinogenic risk. Overall, this paper reveals some possible networks involved in hepatic arsenic-related genotoxicity, carcinogenesis and diabetogenesis. Additional studies to explore further the potential implications of each candidate gene are of especial interest. The present work opens the door to new prospects for the study of i-As mechanisms taking place in the liver under chronic settings.

1. Introduction

Inorganic arsenic (i-As) is an important environmental toxicant broadly spread in water, soil and air. It is known to be cytotoxic, genotoxic and carcinogen in humans [1], but millions of people worldwide are still chronically exposed, despite the recognized associated health risk via highly contaminated drinking water [2]. Many literatures are available associating human i-As exposure with several diseases including anemia, neuropathies, hyperpigmentation or tissue irritations. In the case of chronic exposures i-As causes hyperkeratosis, loss of skin pigmentation, peripheral vascular diseases and, more importantly, an increase in the incidence of diabetes [3] and various cancers of the skin, lung, urinary bladder, liver and kidney [1,4]. Despite the evidences, the mechanisms by which arsenic exerts its toxic and genotoxic effects are not completely known, and the reported data advances a complicated scenario involving different mechanisms acting through cross-related gene networks. Among the proposed potential routes of action we found oxidative DNA damage promotion, acquired tolerance to apoptosis and enhanced cell proliferation, altered DNA methylation and genomic instability, aberrant estrogen signaling, gene amplification or chromosomal abnormalities [4,5]. Some of the mentioned modes of action are predominantly genotoxic while others seem to be more non-genotoxic, and dose–response relationships at low arsenic doses (equivalent to <50 ppb in the drinking water supply of human beings) remain unclear.

After i-As exposure, arsenic is biotransformed and eliminated through urine following a standard profile which in humans consist in: 10–30% of i-As (As^{III} and As^{V}), 10–20% of MMA ($MMA^{III} + MMA^{V}$) and 60–80% of DMA ($DMA^{III} + DMA^{V}$) [6]. The toxicity and carcinogenic potential of intermediate organic metabolites, such as monomethylarsonous acid (MMA^{III}), has been found to be much

* Corresponding author at: Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Edifici Cn, Campus de Bellaterra, 08193 Cerdanyola del Vallès, Spain.
E-mail address: ricard.marcos@uab.es (R. Marcos).

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higher than their inorganic parental species, both *in vitro* and *in vivo* [7,8], demonstrating that there is a straight correlation between i-As metabolism, toxicity and the carcinogenic risk of arsenicals. Thus, an important consideration for arsenic-related *in vivo* studies is the use of experimental animal models similar to humans in terms of i-As biotransformation ability. Notably, arsenic is distributed in organs and excreted into urine in manners highly dependent on animal species. According to Aposhian and Aposhian, the hamster and the rabbit are the more appropriate models for human arsenic metabolism [9]. Like in humans, in hamster arsenic is distributed from blood to organs more rapidly and extensively than in other species [10]. Also similar to human, approximately 60% of the ingested arsenic is excreted through hamster urine in the first following days [11] and, on the basis of MMA excretion, their methylation rate seems to be lower than in other mammals; approximately 17% of the total arsenic is excreted in urine in the form of MMA 24–48 h following arsenite ingestion [12]. Hence, arsenic metabolism in human is more precisely reflected to that occurs in hamster than in other animals. Noteworthy, hamster has proven to be a very accurate model for assessing the associated-arsenic risk during prenatal development [13]. Besides the species-related differential metabolism of arsenic, the route of exposure and the dose range are other important factors to take into consideration when using animal models for risk assessment of arsenic human exposure. To address these concerns, we evaluated the expression changes occurring in the liver of chronically i-As exposed hamsters using low doses and a biologically plausible route.

The use of expression arrays has become a popular technique for gene expression screening. However, this strategy generates large amount of data which is sometimes difficult to interpret. Alternatively, DD-PCR is a robust and reproducible method for identifying differentially expressed mRNAs [14]. As it does not require any prior knowledge in mRNA sequences, it is possible the use of species with not completely annotated genomes or whose arrays are not commercially available.

The study of arsenic carcinogenesis mechanisms is particularly important for human health interests, liver being an important arsenic target. Epidemiology studies have clearly indicated an association between chronic arsenic exposure and preneoplastic lesions such as abnormal liver function, hepatomegaly, hepatoportal sclerosis, liver fibrosis and cirrhosis. The association between environmental arsenic exposure and human liver cancers has been also repeatedly reported [4]. Although tumor formation was not evaluated in the present work, the *in vivo* MN test was used as a biomarker of the genotoxic/carcinogenic risk of the i-As exposure. The MN test has been extensively used as a pertinent tool for evaluating the exposure effects to genotoxic agents. It enables to detect chromosome fragments (clastogenicity) or whole chromosome loss (aneuploidy), both effects being of great relevance in carcinogenesis [15]. Noteworthy, Bonassi et al. provided evidence indicating that increased MN frequency predicts the risk of cancer in humans [16].

It is probable that multiple mechanisms are involved in arsenic-induced hepatic toxicity, genotoxicity and carcinogenesis, some of which may be specific to the liver. Overall, this paper is an attempt to identify genes potentially involved in i-As hepatic mechanisms through the analysis of differentially expressed genes in the liver of hamster after chronic low-arsenic exposure.

2. Materials and methods

2.1. Animals

Golden Syrian hamsters (5–6 weeks, ~100 g) were purchased from Janvier (Le Genest, St. Isle, France) and acclimated for at least 2 weeks prior to experimentation in an environmentally controlled animal facility at the *Universitat Autònoma de Barcelona* (UAB), operating on a 12 h/12 h dark/light cycle and at 20–24 °C. Animals were kept in 4–5 animal cages and provided with an adequate hamster diet and water *ad libitum* throughout the acclimation and experimentation. The study was approved by the Ethic Committee at the UAB.

Individual animal weights, water and food consumptions were recorded bi-weekly for the complete duration of the experiment. Animals were checked daily for signs of morbidity by the facility veterinarian.

2.2. Arsenic treatment and sample collection

A total of 16 hamsters were divided into treated ($n = 11$) and control ($n = 5$) groups. The treated group received drinking water *ad libitum* containing 15 mg i-As/L (~1 mg i-As/kg/day) as sodium arsenite (27 mg $NaAsO_2$/L; pH = 7.5, freshly prepared every two days) for 18 weeks. The control group was kept under the same conditions and received the same drinking water, except for the arsenic content. Blood was collected from the animals at different points of treatment for immediate analysis of micronucleated reticulocytes (MN-RETs). After 18 weeks, animals were euthanized by guillotine. The livers were excised, immediately frozen on dry ice and further stored at −80 °C until use. Approximately 1 mL of blood was also obtained from cardiac punction and kept at room temperature into ethylene diamine tetra acetic acid (EDTA) tubes for MN-RET scoring within the next 24 h.

2.3. In vivo erythrocyte micronucleus test

The protocol developed by Hayashi et al. with some modifications was followed for the scoring of MN-RET in peripheral blood of arsenic-treated and control animals over the 18 weeks of treatment [17]. Briefly, 10 μL of fresh blood was fixed with 30 μL of 10% Carnoy (3 methanol:1 glacial acetic acid). Then, 10 μL of 500 μg/mL acridine orange was added to 10 μL of the mixture, and smears were immediately made on pre-cleaned and pre-heated microscope slides subsequently covered with 24 mm × 50 mm coverslips and kept at room temperature for immediate MN-RET scoring. Two different slides were prepared for each animal. The frequency of MN-RET was blind-scored in an Olympus BX50 fluorescent microscope through an oil immersion objective and using a filter of 515 nm. 1000 RETs were scored per slide. Mitomycin C (MMC; Sigma–Aldrich) was dissolved with sterile distilled water and used at a dose of 2 mg/kg i.p. for 24 h as a positive control for the MN-RET assay at the treatment-time-point weeks 1 and 12. The values obtained were 25.7 ± 2.51 and 25.7 ± 4.04 MN/1000 RETs ($n = 3$), respectively, whereas the corresponding negative controls given physiological saline solution i.p. showed 4.67 ± 0.58 and 4.54 ± 1.52 MN/1000 RETs ($n = 3$), indicating the adequateness of our experimental procedure.

2.4. Total RNA extraction

Total RNA was obtained by the homogenization of approximately 50 mg of liver tissue in 1 mL of TRIzol Reagent (Invitrogen, USA) following supplied indications. RNA integrity was ensured by visualization of 18 and 28S rRNA in an agarose gel electrophoresis (100 V, 30 min at 4 °C; 0.5 μg/mL ethidium bromide) and treated with *DNase I* to eliminate DNA contamination (DNA-free™ kit; Ambion, UK). Total RNA concentration was measured before and after *DNase I* treatment using the NanoDrop™ 2000 (Thermo Fischer Scientific, DE, USA). Only samples with an A_{260}/A_{280} ratio between 1.8 and 2.1 were considered acceptable for further analyses.

2.5. DD-PCR analysis

The reviewed differential display PCR original protocol developed by Liang and Pardee [18] was used for detection of arsenic-induced expression changes with some modifications. Briefly, combinations of 3 different oligo-dT primers (*Anchors* 1–3) and 24 different 5′ random primers (*AP* 1–24) were used for the cDNA synthesis and DD-PCR (for primer sequences, see Table 1) using an iCycler™ Thermal Cycler (Bio-Rad Laboratories, CA, USA). First, total RNA (25 ng) from treated and control livers were reverse transcribed using the Sensiscript RT kit (Qiagen Inc., CA, USA) following supplied instructions. Briefly, 25 ng of RNA was incubated at 37 °C during 60 min in a 20 μL volume reaction containing 0.5 mM of dNTPs, 10 U of RNaseH Inhibitor (Qiagen Inc., CA, USA), 10 U of Sensiscript retrotranscriptase enzyme and 1 μM of the corresponding *Anchor* primer. The DD-PCRs were prepared as follows: 2 μL of the above cDNA containing 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 1 U *Taq* polymerase (Qiagen, CA, USA), 0.5 μM of the previously used *Anchor* primer and 1 μM of the *AP* primer in a 25 μL final volume. Cycling conditions consisted in an initial denaturation step at 94 °C for 4 min, followed by 40 cycles of 30 s at 94 °C, 2 min at 40 °C and 30 s at 72 °C. A final extension step was also carried out during 5 min at 72 °C. Subsequently, the PCRs were mixed with denaturing sequencing dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) and 10 μL aliquots were resolved on standard 8% polyacrylamide (29 polyacrylamide: 1 bisacrylamide; ApliChem GmbH, Darmstadt, Germany), 8 M urea (Sigma–Aldrich, MO, USA) sequencing gels. The electrophoresis was run for 4 h at 2000 V with the power electrophoresis Power-PAC 3000 (Bio-Rad Laboratories, CA, USA), and stained with 2 g/L $AgNO_3$ (Merk, Darmstadt, Germany) following standard protocol. Gels were then dried for band pattern analysis.