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Association between the polymorphism of three genes involved in the methylation and efflux of arsenic (As3MT, MRP1 and P-gp) with lung cancer in a Mexican cohort

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Abstract

Background: Lung cancer is the most common neoplasm and the first cause-related mortality in developed and in most of non-developed countries. Epidemiological studies have demonstrated that even at low arsenic doses, the lungs are one of the main target organs and that chronic arsenic exposure has been associated with an increase in lung cancer development. Among the risk factors for cancer, arsenic methylation efficiency (As3MT) and the clearance of arsenic from cells by two members of the ATP-binding cassette (ABC) transporter family [multidrug resistance protein 1 (MRP1) and P-glycoprotein (P-gp)] play an important role in processing of arsenic and decreasing its intracellular levels.

Objective: Evaluate the association between chronic exposure to arsenic with polymorphism of three proteins involved in arsenic metabolism and efflux of the metalloid in subjects with lung cancer.

Results: The polymorphism of As3MT, MRP1 and P-gp modified the arsenic metabolism increasing significantly the As^V urinary levels. A significant association between MRP1 polymorphisms with an increase in the risk for cancer was found.

Conclusions: The high inorganic arsenic urinary levels registered in the studied subjects suggest a reduction in the efficiency of As3MT, MRP1 and P-gp firstly because of gene polymorphisms

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and secondarily because of high internal inorganic arsenic levels. MRP1 polymorphism was associated with two fold increase in the risk of lung cancer.

Short abstract:

The aim of the study was to evaluate the association between chronic arsenic exposure with the polymorphism of three proteins involved in arsenic metabolism and efflux in subjects with lung cancer. The results suggest a reduction in the efficiency of As3MT, MRP1 and P-gp and MRP1 polymorphism which was associated a two fold increase in the risk of lung cancer.

Keywords

Lung cancer; arsenic; As3MT; MRP1; P-gp; polymorphism

INTRODUCTION

Lung cancer is the most common neoplasm and the first cause-related mortality in developed and in most of non-developed countries. About 1.6 million people die of lung cancer each year and the overall 5-year survival rate is only 15% (WHO, 2010). The etiologic factors of lung cancer is complex and the available data indicate that smoking is the most correlated cause of lung cancer. However, less than 25% of smokers develop the disease in their lifetime, which suggests that there are significant differences in the individual's susceptibility to develop lung cancer (IARC, 2004). Thus additional risk factors must exist such as genetic and environmental factors which must be taken in account to explain the etiology of this cancer.

Among the environmental risk factors, inorganic arsenic (As) stands out because it is a well-known human carcinogen that potentially affects ~160 million people worldwide via primary exposure through the domestic water supply and secondary exposure through food consumption. Epidemiological studies have demonstrated that even at low arsenic doses, the lungs are one of the main target organs (Hubaux et al. 2013), and that chronic arsenic exposure has been associated with an increase in lung cancer development (Straif et al. 2009; NRC 2001). Arsenic-induced malignant transformation is related primarily to the biomethylation (Drobná et al. 2010), as well as, to the clearance process of this metalloid from cells.

Arsenic methyltransferase (As3MT) is the primary enzyme involved in arsenic metabolism and in the clearance process members of the ATP-binding cassette transporter family [P-glycoprotein (P-gp/ABCB1/MDR1), and multidrug resistance protein 1 (MRP1/ABCC1)] play an important role in that process acting as drug efflux pumps which decrease the intracellular levels of arsenic.

The primary enzyme involved in arsenic metabolism is As3MT which converts inorganic arsenic to mono- and di-methylated arsenic species. While this enzymatic process has long been considered as the major route for detoxification, current studies indicated that biomethylation of inorganic arsenic is a process that activates arsenic as a toxin and a carcinogen. Some studies have shown that the methylation process increases the rate of

whole-body arsenic clearance because the methylated species are more efficiently excreted by the cellular efflux proteins. On the other hand, the inorganic arsenic species are not efficiently excreted, which could contribute to their intracellular accumulation. Arsenic methylation efficiency is partly explained by genetic variations in the As3MT gene which are linked to arsenic-related disease susceptibility (Gresner et al. 2007).

Members of the ABC transporter family, particularly, P-gp and MRP1, are expressed in normal lung tissue and in lung tumors and play a very important role in clearance of toxicants from the lung. The prominent expression of P-gp and MRP1 in the human lung suggests that these transporters may be pivotal in protecting the lungs against endogenous or exogenous toxic compounds (Scheffer et al. 2002). These transporters are used by inorganic arsenic metabolism products (Long et al. 2011; Song et al. 2010) by means of it efflux across the plasma membrane as arsenic triglutathione and as diglutathione conjugates of monomethylarsonous acid (Banerjee et al. 2018) or sequestering the toxic compounds within intracellular organelles as thiol conjugates (Leslie et al. 2004). Thus, these efflux proteins contribute to the many physiological and pathophysiological processes influenced by these compounds, including oxidative stress. It is still unclear, to which extent these efflux pump transporters (P-gp and MRP1) contribute to arsenic clearance. In a previous study, we reported a negative correlation between MRP1 expression and chronic arsenic exposure in subjects with lung cancer (Recio-Vega et al. 2015). Several studies evaluating the effects of polymorphism of both efflux pumps on their function have been reported. In fact, recently in a study carried out in 112 mother-placenta pairs to detect whether maternal P-gp polymorphism has an effect on placental arsenic levels, it was shown that that fetuses of mothers with TT genotype may be more susceptible to arsenic toxicity as compared to those of with CC and CT genotypes (Kaya-Akyuzulu et al. 2016).

Because there are no reports associating the polymorphisms of the primary enzyme involved in arsenic metabolism (As3MT) and of the two of the most important proteins involved in the cellular efflux of arsenic (MRP1 and P-gp) with the urinary arsenic levels in lung cancer subjects who have been chronically exposed to arsenic through drinking water, the present study was designed.

MATERIAL AND METHODS

Study population

A cross sectional study was carried out and participants were recruited from Department of Respiratory Diseases, Mexican Institute of Social Security, Torreon, Coahuila, Mexico. The volunteers were residents of the Comarca Lagunera where higher arsenic tap water levels have been detected in the last 32 years, ranging from 10.4-360 ppb in 2015 (Recio-Vega et al., 2015). The geographic area received groundwater through the local water supply and the high As levels in the water are due, in part, to an over-extraction of water from the ground for crops. At present, water is obtained from a depth of 200-300 meters. The Comarca Lagunera, is located in the north-central part of Mexico and known to be an area where increased arsenic toxicity has been reported (Sampayo-Reyes et al., 2010; Recio-Vega et al., 2015).

Eligible patients included women and men greater than 18 years old and who had at least 10 years of residence in the Comarca Laguna, Mexico. All patients with other types of cancer or who had received radiotherapy or chemotherapy prior to the study were excluded. A total of 200 subjects were included. The cases were 100 newly diagnosed volunteers with a first diagnosis of lung cancer identified by histopathology from cells obtained through bronchial lavage. The controls were 100 participants with negative cells for malignancy who came from the same hospitals and geographical area. Written informed consent was obtained from each participant. The study protocol was approved by the Ethics Committee of the School of Medicine at Torreon, University of Coahuila, Mexico.

Questionnaire application

Information was collected through in-person interviews and included socio-demographic variables (schooling, socioeconomic status, type of kitchen, the type of fuel used for cooking), lifetime residential history, life style (smoking, alcohol intake and exercise), a detailed family history of cancer (any family member), occupational history, history of As exposure and diet. Water consumption habits were ascertained through a standardized questionnaire [Recio-Vega et al., 2015]. The potential sources of arsenic exposure (industry, agricultural fields, and others) located close to the participants' homes (<1,000 m) were identified and recorded. A history of occupational exposure, with an emphasis on exposure to potential As sources, was scored in a binomial fashion.

Genotyping

As3MT-Met287Thr (rs11191439), P-gp-C3435T (rs2032582) MRP1-Arg723Gln (rs4148356)

Peripheral blood samples were taken into EDTA vacutainer tubes, and extraction of genomic DNA material was carried out with DNAzol according to the manufacturer's instructions. For detection of SNPs TaqMan probes (Applied Biosystems, CA, USA) were used following the supplier's instructions. Briefly, to a cap PCR tube with optical quality, 10 μL of TaqMan Genotyping Mix 0.5 μL trial were added, which included the two primers and fluorescent probe specific for each of the polymorphisms. Two μL was further added to the solution containing the DNA probe (~10-20 ng) and the final volume of 20 μL was completed by adding molecular grade water (all reagents from Applied Biosystems, CA, USA). Thermocycling reactions were performed in a Step-One™ Real Time PCR System (Applied Biosystems, CA, USA) under the following conditions: a cycle of polymerase activation of 95 °C for 10 minutes, then 60 cycles of denaturation at 92 °C for 15 seconds and the alignment/extension at 60 °C for 1 minute. Positive and negative controls were included in the same 96-well plate. Allelic discrimination was developed using the Step-One software system (Applied Biosystem, CA, USA). All SNPs passed the Hardy-Weinberg equilibrium chi-squared test with P-value >0.05.

Genotyping: selection of polymorphism sites by q-PCR

For the detection of single nucleotide polymorphisms (SNPs), TaqMan SNP gentotyping assays (AppliedBiosystems) were used following the supplier's instructions. In a PCR tube with a cap, 10 μl of Taqman Genotyping Master Mix, 0.5 μl of assay, which included the

two primers and the fluorescent probe specific for each of the polymorphisms, 2 μl of the solution containing the DNA (10 to 20 ng) was also added and the final volume of 20 μl was completed by adding water molecular grade (all Applied Biosystems reagents). Thermocycling reactions were performed in an Applied Biosystems Step-One Real Time PCR system. The analysis was performed using the One Step equipment software from Applied Biosystems.

All assay reagents were purchased from Applied Biosystems. The As3MT, MRP1 and P-gp probes and gene-specific primers were used as an endogenous control. The amplification of As3MT, MRP1 and P-gp genes in the same sample normalized the differences in DNA concentration between samples and ensured that no false-negative As3MT, MRP1 and P-gp *0/0 genotypes due to PCR or pipetting failure or insufficient DNA concentrations in the original sample were generated. The initial step of the PCR reaction was set at 95 °C for 10 minutes, and the denaturation step was performed at 50-92 °C for 15 seconds. The amplification was performed at 59-60 °C for 1 minute. PCR was performed (Step-One™ Real Time PCR System; Applied Biosystem, CA, USA) in 96-well plates with a final sample volume of 10 μL, including 1X universal PCR master mix (TaqMan; Applied Biosystems), 25 ng DNA, and proper dilutions of primers according to the manufacturer's instruction. Data were then collected (Absolute Quantification; SDS software, ver. 1.3.1, SDS, Cary, NC). Copy number estimation was conducted by the ΔCt method (Copy-Caller software, ver. 1.0; AB). The calculation was performed by a maximum-likelihood algorithm built into the software [Zhou et al., 2010].

As measurement in drinking water and urine

Drinking water samples (well) were collected from each rural community included in the study and analyzed for inorganic arsenic levels. No other contaminants in the drinking water were assessed. Well water samples from each rural community are representative of the water that participants drink and it is provided through the unique local water supply system. Individual exposure was assessed based on the urinary concentration of the total arsenic level. A first morning void urine sample was collected in sterile 120-mL screw-topped polypropylene containers.

Urine samples were analyzed using the methodology described by the U.S. Center for Disease Control (CDC, 2004) at the Arizona Laboratory for Emerging Contaminants, University of Arizona, Tucson, Arizona, U.S.A. Briefly, arsenic species in urine (As^{V}, As^{III}, monomethylarsonic acid (MMA^{V}), dimethylarsinic acid, (DMA^{V}) and arsenobetaine) were separated by High Performance Liquid Chromatography (HPLC) and analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Arsenic concentrations in urine were analyzed by inductively coupled plasma mass spectrometry utilizing Standard Reference Water, SMR 1640 (NIST, Gaithersburg, MD, USA) and freeze-dried Urine Reference Material for trace elements (Clincheck-control; RECIPE Chemicals instruments GmbH, Munich, Germany) for urine as quality control. Urinary As concentrations were adjusted by urine creatinine levels. Additional exposure to other arsenic compounds, which usually is attributable to consumption of rice, seafood such as bivalves and seaweeds, was considered minimal because such seafood is essentially little eaten in this area. Arsenic

metabolism efficiency was calculated using the following formulas proposed by Del Razo et al (1997): first methylation = $MMA^V/(As^V+As^{III})$; second methylation = DMA^V/MMA^V.

Statistics

Independent and dependent variables were described according to their frequency and distribution measurements (arithmetic mean and standard deviation). The Chi^2 test or F test was used when the variable was divided into more than two categories. According to the data's distribution a dichotomous variable Student's t test or Mann Whitney test was used to compare different genés genotyping variants and/or arsenic levels. This method permitted us to establish statistical differences among groups for each dependent variable. Linear and logistic regression models were used to assess crude or independent associations between patient demographic variables, arsenic and gene polymorphisms, and to establish the odds ratios (ORs), respectively. The ORs for lung cancer risk were estimated using unconditional logistic regression. The covariates were selected for the final models based on either significance ($P<0.05$) or a greater than 10% change in the observed OR. For multiple analyses, we included all relevant variables identified in the bivariate model. The confounding variables included in the final analyses were schooling, cooking with wood, smoking and type of drinking water. For deviation from Hardy-Weinberg equilibrium, chi-square analysis was used. All analyses were performed using the statistical software STATA 11.0 (Stata Corp., College Station, TX).

RESULTS

Table 1 shows the distribution of data on selected characteristics for the subjects. When the socio-demographic variables were compared between the studied groups, there were only two significant differences between them, schooling and years living in the studied area. Controls had higher schooling and more years of residency at the Comarca Lagunera. The mean age of cases was slightly higher, but it was not significantly different to controls' age. Forty two percent of cases drank tap water; meanwhile, only 24% of controls drank it; similarly, the frequency of subjects cooking with wood was higher in cases ($p<0.05$).

When the frequency to several risk factors exposure for lung cancer was compared between cases and controls, the results disclosed that all the evaluated variables related to smoking [smoking history (68% vs 51%), smoking years (26.8 vs 19.2), number of cigarettes smoked by day (16.6 vs 5.7) and smoking index (23.5 vs 5.8)] were significantly higher in the cases. No other factors (alcohol intake, familiar history of lung cancer or other cancers) were different between the studied groups. The environmental exposure to other type of studied toxicants was not different between groups (Table 1).

When the urinary levels of arsenic and its metabolites were compared between cases and controls, only As^V concentration were statistically higher in cases. The number of subjects with As levels >50 ug/L was higher (more than two folds) in cases than in the controls, however, the frequency and its mean urinary arsenic levels were not statistical different (Table 2). No differences were found in total As urinary levels between subjects who drink tap water (26.3 ug/L) vs purified water (21.5 ug/L) or in smoking (23.3 ug/L) vs non-smoking subjects (27.0 ug/L) ($p>0.05$).

It can be seen that the most frequent allele detected is the wild homozygote, followed in frequency by the heterozygote and finally by the mutated homozygote. No differences were found when the frequency of As3MT, MRP1 and P-gp genotyping were compared between cases and controls. When the frequency of homozygous and heterozygous mutant variants of these three genes were combined (wt/vt + vt/vt), the combination of the mutant variants of that gene were more frequently observed in cases. Only the frequency of MRP1's mutant variants were significantly higher in cases than in controls, indeed, the number of these MRP1 abnormal genotypes was 1.5 times more frequently observed in cases (Table 3). The higher polymorphism frequency was observed for the As3MT gene followed by the MRP1 and the lower for the P-gp gen (Table 3).

When the urinary arsenic and its metabolites levels were analyzed and compared intra-group and between the studied groups in the As3MT study, we found that in cases, the level of As^{III} was higher in the heterozygous variant (wt/vt) than in the homozygous mutant variant (vt/vt). When the levels were compared between the studied groups, As^{V} and DMA^{v} were higher in cases' heterozygous variant (wt/vt) than the levels found in controls, as well as, MMA^{v} was higher in the cases' homozygous mutant variant (vt/vt) (Table 4).

In the MRP1 study we found that in cases the total As levels were higher in the combined mutant variants (wt/vt+vt/vt), meanwhile, iAs in the heterozygous variant (vt/vt) and first methylation levels in the heterozygous variant (wt/vt) where lower when compared with the found in the homozygous variant (vt/vt). When the levels were compared between cases and controls, As^{V} and MMA^{v} were higher in cases' combined mutant variants (wt/vt + vt/vt) than in the same genotype variant of controls (Table 5).

Finally, in the P-gp group, we only found statistical differences when As or its metabolites levels were compared between groups. As^{v} concentration was higher in cases' homozygous variant (wt/wt) and in the combination of mutant variants (vt/vt+ wt/vt) than the levels found in the same variants from controls. Similarly, the first methylation levels were higher in the cases' homozygous mutant variant (vt/vt) (Table 6). Generally, in the three analyzed genes, the higher urinary levels of As or its metabolites were observed in the abnormal genotypes (wt/vt and/or vt/vt) (Tables 4–6).

When the association between lung cancer and the different studied variables in the non-adjusted linear regression model were evaluated, significant associations ($p<0.05$) were found with schooling, residency, years of residency, BMI, smoking, number of cigars smoked per day, smoking index, cooking with wood, type of drinking water, economic income, and with MRP1 polymorphism. When the statistical model was adjusted, the associations which remained significant were smoking index and MRP1 polymorphism (Table 7). No association were found with As or its species.

When the studied subjects were divided according with their arsenic urinary levels (≤50 ug/L and >50 ug/L), the only significant association found with lung cancer was with the MRP1 polymorphism (β 0.050; 95% CI 0.000 - 0.327) in volunteers with arsenic levels ≤50 ug/L.

When the risk for lung cancer was calculated (OR), only smoking index and MRP1 polymorphism were statistically significant (Table 8).

DISCUSSION

Because lung cancer is the most common cancer, the first cause-related mortality in the world, and its etiology and development of is complex, there are significant differences in an individual's susceptibility. Thus it is relevant to assess conjointly the relationship between genetic and environmental factors on the cancer development.

In our study all variables related to smoking were significantly higher in the cases making the smoking index significantly associated with lung cancer. Another important risk factor found in our study was the fact that almost twofold of cases drank tap water for more than 38 years which was contaminated with arsenic at more than two time the WHÒs public health concern level (>23 ug/L vs 10 ug/L). This metalloid is a well-known human carcinogen, and chronic exposure to it has been associated with an increase in lung cancer development (Straif et al. 2009; NRC 2001). Therefore, in the studied subjects the exposure to high arsenic water level and the long-term exposure to it could contribute to their cancer development. The urinary national concern total arsenic levels (>50 ug/L) were higher in cases (more than two folds) than in the controls with the frequency and mean urinary arsenic levels at the borderline statistical difference (p=0.053). When we compared urinary arsenic and its species levels between cases and controls, only the inorganic As^V concentration was statistically higher in cases. These results were not consistent with the results obtained by our group in a previous study (Recio-Vega 2015), in which MMA^V, DMAV, %DMAV, and the first methylation index were lower ($P < 0.05$), and As^V and total iAs were higher ($P < 0.05$), in the positive cases than in the controls. These data suggested a low overall excretion rate and arsenic retention in tissues, which could increase the risk for carcinogenesis. It is known the inorganic arsenic species are not efficiently excreted; which increase their intracellular accumulation propitiating the production of ROS and hence generating oxidative stress (Shi et al. 2004) and/or inhibiting DNA damage repair; which can induce cancer development. Arsenic methylation efficiency (a susceptibility factor for arsenic toxicity) is another factor that has been described as a risk factor for cancer. The first and second methylation values were lower in cases than in controls; however, this difference was not statistically significant. The methylation efficiency is partly explained by genetic variations in the As3MT gene (inherited genetic predispositions related to the genetic polymorphisms) which are linked to arsenic-related disease susceptibility (Gresner et al. 2007). We evaluated and compared the frequency of the As3MT polymorphism (rs11191439). The mutant variants were slightly higher in cases, but it was not significant. When the arsenic species levels were compared between groups according with their genotyping, As^V and MMA^V levels were significantly higher in the cases carriers of the As3MT gene variants, which suggests that the gene variants modify arsenic metabolism. Similar to our results, De Lomas et al. (2018) evaluated the associations between As3MT polymorphisms and arsenic methylation efficiency and found higher %iAs and %MMA, and lower %DMA in urine, among rs1046778 TT carriers compared to CC carriers. Antonelli et al. (2014) concluded that their data support the hypothesis that As3MT polymorphisms alter *in vivo* metabolite concentrations. In another clinical study, De la Rosa

et al. (2017) evaluated whether As3MT and N6AMT1 gene polymorphisms alter arsenic methylation and impact iAs-related cancer risks and concluded that there exists a direct association between As3MT polymorphisms and arsenic-related internal cancer risk. In an experimental study, the distribution and retention of arsenic were compared in adult female As3mt knockout mice and wild-type C57BL/6 mice. The authors found a lower urinary concentration of arsenic and higher fractions of the body burden of arsenic in liver, kidney, and urinary bladder in As3mt knockout mice than in wild-type mice. These organs and lung had significantly higher arsenic concentrations than corresponding organs from wild-type mice. Inorganic arsenic was the predominant species in tissues of As3mt knockout mice. Diminished capacity for arsenic methylation in As3mt knockout mice prolongs retention of inorganic arsenic in tissues and affects whole body clearance of arsenic. Altered retention and tissue tropism of arsenic in As3mt knockout mice could affect the toxic or carcinogenic effects associated with exposure to this metalloid or its methylated metabolites (Hughes et al. 2010). According with our data and with the results from the previously mentioned studies, the As3MT polymorphisms induce disruption of arsenic metabolism increasing the non-methylated species and the risk for cancer development. In order to prevent and/or decrease the incidence of arsenic-related diseases, it so important to detect which subjects exposed to arsenic carry an abnormal As3MT genotype.

Although multiple pathways are responsible for the inter-individual differences in arsenic metabolism, the arsenic efflux proteins play an important role because different members of the ATP-binding cassette transporter superfamily subfamily “C” (MRP1, MRD1 and others) are critical for protecting many organisms from arsenic (Long et al. 2011; Song et al. 2010). This protein reduces arsenic toxicity either by extruding multiple methylated and/or glutathionylated metabolites of arsenic (Banerjee et al., 2014; Shukalek et al., 2016) from the cells or sequestering it within intracellular organelles as thiol conjugates (Leslie et al. 2004). MRP1 is expressed in most tissues throughout the body, with relatively high levels found in the lungs, testes, and kidneys (Drobná et al. 2010). In the lungs, MRP1 is highly expressed at the basolateral membrane of human bronchial epithelial cells (Bréchot et al. 1998). The prominent expression of P-gp and MRP1 in the human lung suggests that these transporters may be pivotal in protecting the lungs against endogenous or exogenous toxic compounds (Scheffer et al. 2002). Alteration of MRP1 expression has been associated with to the susceptibility to certain lung diseases (Okamura et al. 2013). Inherited genetic predispositions related to the genetic polymorphisms of proteins involved in the phases 1-3 of xenobiotic metabolism are believed to underlie this susceptibility. MRP1 is highly polymorphic and genetic variants could account for some of the well-established but poorly understood interindividual susceptibility to arsenic-induced carcinogenesis (Conseil et al. 2005; Hernandez et al. 2008). Efflux of different arsenic metabolites by MRP1 is likely influenced by multiple factors, including cell and tissue type and according with the results obtained previously by our group in subjects with lung cancer, another factor that modifies MRP1 efficiency is exposure to high arsenic levels. Indeed, we found that the grade of MRP1 expression in bronchoalveolar lavage cells was significantly diminished in subjects with high arsenic urinary levels (>50 μg/L) (Recio-Vega 2015).

In the present study, we evaluated one of the most frequent described polymorphisms of two proteins that have been related with arsenic efflux from cells (MRP1 and for

P-gp) and we found that the frequency of MRP1's mutant variants and that the As^V and MMA^v levels were significant higher (1.5, 1.8 and 1.3 times, respectively) in cases than in controls. Similarly, in the carriers of the mutant variants of P-gp, As^V levels were higher in cases. In accordance with our results, As^V urinary levels were significantly higher in cases which carry As3MT, MRP1 and P-gp polymorphisms. This fact is relevant because as we commented previously, the inorganic arsenic species are not efficiently excreted and increase the production of ROS generating oxidative stress (Shi et al. 2004) and/or inhibiting DNA damage repair; which could induce cancer development. Therefore, tissue-specific susceptibility to arsenic-induced disease and tumor sensitivity to arsenic can be influenced by each of these elements that intervene in the cellular metabolism of the metalloid.

Once inside the cell, As3MT induces the reduction of As^V to As^{III} and then methylation (Cullen, 2014) and glutathionylation pathways of arsenic to produce DMA^V as end product. As3MT polymorphisms induce disruption of arsenic metabolism increasing the non-methylated species (As^V) and this negatively influences the methylation and glutathionylation of arsenic and subsequently altering the metabolites available for MRP1 and P-gp export. Inorganic arsenic species (As^V and As^{III}) must be converted to glutathionylated compounds ($As(GS)_3$) prior its efflux by MRP1 (Leslie et al., 2004; Banerjee et al. 2018). It has been reported that chronic exposure to high arsenic concentration and the high cellular stress induced by cancer, could deplete GSH intracellular levels. Kojima et al. (2016) mentioned that the depletion of cellular GSH and the inhibition of Mrps and P-gp functions increased cellular arsenic uptake and reduced arsenic tolerance in cells. According with our results, the polymorphism of As3MT, MRP1 and P-gp modified at least two steps of the arsenic metabolism (methylation and efflux) increasing significantly the As^V internal dose and subsequently its urinary levels. The polymorphism of these genes, suggest an abnormal efficiency which have a substantial impact on the development and/or progression of the disease. Another important point regarding the high arsenic internal (intracellular) levels is that at low levels of arsenic exposure, MRP1 is potentially important for the export of As(GS)3 and any DMA^V that is formed (preventing the formation of the highly toxic DMA^{III}) which prevent product inhibition of As3MT (Banerjee et al 2018); however, this physiological process could be negatively influenced by the high arsenic levels of exposure in the studied subjects, which in turn would increase the inefficiency of the studied genes and subsequently the risk for cancer development.

An important finding of our work was the positive association between the MRP1 polymorphism with lung cancer and the increased risk for this neoplasm in the carriers of this polymorphism. It is important to remark that MRP1 is an important detoxification pathway because it transports in addition to the methylated and/or glutathionylated metabolites of arsenic, a variety of xenobiotics, as well as, physiological compounds including GSH, glutathione disulphide, 17β-estradiol 17-(β-D-glucuronide) (E217βG), and leukotriene C4 (LTC4) (Cole SP. 2014) preventing the accumulation of compounds in cells and in tissues. Therefore, in the carriers of this polymorphism in addition of the metalloid species, a variety of chemical compounds could be accumulated into cells increasing the risk for cancer development/progression.

Further studies are required for continued investigation into the potential use of As3MT, MRP1 and P-gp polymorphisms as biomarkers for lung cancer and in how the cellular environment influences the function of MRP1, specifically for arsenic species cell detoxification.

In summary, the high inorganic arsenic urinary levels registered in the studied subjects suggest a reduction in the efficiency of As3MT, MRP1 and P-gp firstly by its gene polymorphism and secondarily to the high internal inorganic arsenic levels which could reduce its tissue expression or inhibiting them. Therefore, the synergetic relationship between the polymorphic genes and high chronic arsenic exposure potentially increased the susceptibility in the studied population for lung cancer development. Finally, MRP1 polymorphism was associated to lung cancer and in its carriers the risk for cancer was increased in more than twofold.

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Table 1.

Socio-demographic and anthropometric variables of the studied population. Results are showed as frequency or arithmetic mean and standard deviation (range).

	Cases (n=100)	Controls (n=100)	P value
Age (years)	64.1 ± 13.8 (21-91)	60.3 ± 14.5 (24-88)	0.059
BMI *	24.3 ± 5.2 (13-48)	25.6 ± 4.6 (16-37)	0.06
Schooling (years)	6.1 ± 5.3 (0-20)	8.7 ± 6.6 (0-24)	**0.002**
Residency			
Urban	71 (71%)	79 (79%)	0.192
Rural	29 (29%)	21 (21%)	
Years living at Comarca Lagunera	38.4 ± 24.5	45.8 ± 23.5	**0.03**
Smoking history	68 (68%)	51 (51%)	**0.014**
Smoking index	23.5 ± 28.5	5.8 ± 12.6	**0.000**
Type of drinking water			
Tap water	42 (42%)	24 (24%)	**0.013**
Purified	47 (47%)	55 (55%)	
Both	11 (11%)	21 (21%)	
Cooking with wood	32 (32%)	18 (18%)	**0.022**
Sea food intake	75 (75%)	83 (83%9	0.306
Living near to an industry			
Fuel station	80 (80%)	28 (28%%)	0.185
Brick factory	1 (1%)	5 (5%)	0.097
Cement factory	7 (7%)	12 (12%)	0.228
Metallurgic factory	14 (14%)	12 (12%)	0.674

$p<0.05$: t test, Mann-Whitney or Chi^2.

*IMC weight/height^2.

Smoking index; was calculated as: (number of cigarettes per day) (number of smoking years)/cigarettes per pack.

Table 2.

Urinary levels of arsenic and its metabolites (ug/L) in the studied subjects. Results are shown as arithmetic mean and standard deviation (range).

	Cases (n=100)	Controls (n=100)	P value
Total As	26.3 ± 26.2 (0.0-164.3)	23.2 ± 25.0 (0.0-180.2)	0.39
As^{III}	2.6 ± 2.5 (0.0-17.0)	3.1 ± 3.8 (0-22.0)	0.35
As^{V}	1.5 ± 2.40 (0.0-14.6)	1.0 ± 1.4 (0.0-8.3)	**0.017**
iAs	3.0 ± 3.2 (0.0-16.2)	3.3 ± 4.5 (0.0-24.2)	0.62
MMA^{V}	4.7 ± 4.6 (0.1-22.3)	3.89 ± 4.8 (0.0-33.3)	0.09
DMA^{V}	20.4 ± 20.6 (0.3-139.4)	17.0 ± 16.9 (1.2-123.7)	0.36
First methylation	1.8 ± 2.32 (0.0-18.5)	3.0 ± 7.1 (0.2-48.0)	0.94
Second methylation	5.7 ± 4.3 (1.6-29.9)	6.1 ± 5.6 (1.7-50.8)	0.60
Total As			
≤50	83 (83%) 17.0 ± 13.1	92 (92%) 17.3 ± 13.0	0.05
>50	17 (17%) 71.3 ± 26.9	8 (8%) 81.6 ± 40.0	

p<0.05: Student's t test or Mann-Whitney

Table 3.

Frequency of As3MT, MRP1 and P-gp polymorphism genotyping. Results shown as frequency (percent).

Gene	Cases (n=100)	Controls (n=100)	P value	Alelic Frequency
As3MT				
wt/wt	71 (71)	80 (80)	0.10	0.16
wt/vt	17 (l7)	16 (16)	0.13 *	
vt/vt	12 (12)	4 (4)		
wt/vt + vt/vt	29 (29)	20 (20)		
MRP1				
wt/wt	67 (67)	80 (80)	0.08	0.13
wt/vt	25 (25)	17 (17)	0.037 *	
vt/vt	8 (8)	3 (3)		
wt/vt + vt/vt	33 (33)	20 (20)		
P-gp				
wt/wt	84 (84)	86 (86)	0.53	0.07
wt/vt	13 (13)	9 (9)	0.692 *	
vt/vt	3 (3)	5 (5)		
wt/vt + vt/vt	16 (16)	14 (14)		

$p < 0.05$ Chi^2 (wt/wt *vs* wt/vt; vt/vt);

* $p<0.05$ Chi^2 (wt/wt *vs* wt/vt; vt/vt; wt/vt + vt/vt).

Alelos: wt/wt = wild type; wt/vt and vt/vt= variant types.

Table 4.

Urinary As levels by the As3MT genotyping. Results are shown as arithmetic mean and standard deviation.

	Cases				Controls			
As3MT	**wt (n=71)**	**wt/vt (n=17)**	**vt/vt (n=12)**	**wt/vt+vt/vt (n=29)**	**wt (n=80)**	**wt/vt (n=16)**	**vt/vt (n=4)**	**wt/vt+vt/vt (n=20)**
Total As	25.1 ± 26.8	31.2 ± 27.0	24.3 ± 21.8	28.3 ± 24.8	24.6 ± 27.1	17.8 ± 13.1	9.8 ± 5.0	16.2 ± 12.3
As^{III}	**2.5 ± 1.8** *a*	**3.6 ± 2.6** *a*	1.7 ± 1.1	2.7 ± 2.3	3.2 ± 4.1	2.4 ± 2.4	1.4 ± 1.5	2.2 ± 2.2
As^{V}	1.2 ± 1.3	0.9 ± 0.7	**4.9 ± 6.4** *b*	2.1 ± 3.8	1.1 ± 1.65	0.5 ± 0.4	**0.5 ± 0.4** *b*	0.4 ± 0.4
MMA^{V}	4.4 ± 4.54	**6.7 ± 5.8** *b*	4.2 ± 2.7	5.6 ±4.8	4.2 ± 5.2	**2.7 ± 2.6** *b*	1.6 ± 1.0	2.5 ± 2.4
DMA^{V}	20.3 ± 21.7	22.8 ± 19.3	**17.8 ± 16.0** *b*	20.8 ±17.9	18.5 ± 18.2	12.5 ± 9.3	**6.7 ± 3.5** *b*	11.4 ± 8.7
iAs	2.7 ± 2.6	3.8 ± 3.2	4.0 ± 5.6	3.9 ± 4.3	3.6 ± 4.9	2.4 ± 2.5	1.3 ± 1.2	2.2 ± 2.3
First methylation	1.8 ± 2.4	1.4 ± 1.0	2.2 ± 2.7	1.7 ± 2.0	3.2 ± 7.7	1.5 ± 1.0	5.7 ± 8.9	2.3 ± 4.0
Second methylation	6.0 ± 4.7	5.2 ± 3.9	4.6 ± 2.5	5.0 ± 3.3	6.2 ± 6.2	6.1 ± 3.2	4.5 ± 2.7	5.8 ± 3.21

a,b $p<0.05$: Mann-Whitney.

a comparison intra-group (wt/wt *vs* wt/vt and vt/vt and wt/vt+vt/vt).

b comparison between cases and controls (wt/wt *vs* wt/wt; wt/vt *vs* wt/vt; vt/vt *vs* vt/vt and wt/vt+vt/vt *vs* wt/vt+vt/vt).

Alelos: wt/wt = wild type (homozygous), wt/vt= heterozygous.

Table 5.

Urinary As levels by MRP1 genotyping. Results are shown as arithmetic mean and standard deviation.

	Cases				Controls			
MRP1	**wt (n=67)**	**wt/vt (n=25)**	**vt/vt (n=8)**	**wt/vt+vt/vt (n=33)**	**wt (n=80)**	**wt/vt (n=17)**	**vt/vt (n=3)**	**wt/vt+vt/vt (n=20)**
Total As	**25.2 ± 29.6** *a*	25.8 ± 14.4	37.1 ± 24.3	**28.6 ± 17.6** *a*	23.2 ± 26.5	23.4 ± 19.1	12.2 ± 10.1	21.7 ± 18.2
As^{III}	2.5 ± 2.8	2.8 ± 1.9	2.4 ± 1.8	2.7 ± 1.9	3.0 ± 3.4	3.5 ± 5.4	2.4 ± 1.9	3.4 ± 5.3
As^{V}	1.5 ± 2.8	1.2 ± 1.1	1.8 ± 2.0	**1.3 ± 1.2** *b*	1.0 ± 1.6	0.7 ± 0.5	0.7 ± 0.5	**0.7 ± 0.5** *b*
MMA^{V}	4.8 ± 5.1	4.1 ± 3.0	6.0 ± 5.3	**4.6 ± 3.7** *b*	3.9 ± 5.2	3.8 ± 2.9	1.6 ± 1.3	**3.4 ± 2.8** *b*
DMA^{V}	20.4 ± 23.7	17.9 ± 11.4	28.4 ± 7.2	20.5 ± 13.5	17.3 ± 18.1	17.0 ± 11.2	9.11 ± 7.1	15.7 ± 10.9
iAs	**2.8 ± 3.4** *a*	**3.7 ± 2.6** *a*	2.5 ± 3.0	3.4 ± 2.7	3.2 ± 4.4	3.7 ± 5.6	1.5 ± 1.7	3.4 ± 5.2
First methylation	**1.8 ± 2.5** *a*	1.3 ± 0.8	**3.4 ± 3.0** *a*	1.7 ± 1.7	3.4 ± 7.8	1.3 ± 0.7	1.6 ± 0.7	1.4 ± 0.7
Second methylation	5.8 ± 4.6	5.3 ± 4.1	6.4 ± 3.6	5.5 ± 4.0	6.3 ± 6.2	5.1 ± 2.2	7.8 ± 5.2	5.5 ± 2.9

a,b $p<0.05$: Mann-Whitney.

a comparison intra-group (wt/wt *vs* wt/vt and vt/vt and wt/vt+vt/vt).

b comparison between cases and controls (wt/wt *vs* wt/wt; wt/vt *vs* wt/vt; vt/vt *vs* vt/vt and wt/vt+vt/vt *vs* wt/vt+vt/vt).

Alelos: wt/wt = wild type (homozygous), wt/vt= heterozygous.

Table 6.

Urinary As levels by P-gp genotyping. Results are shown as arithmetic mean and standard deviation.

	Cases				Controls			
P-gp	**wt (n=84)**	**wt/vt (n=13)**	**vt/vt (n=3)**	**wt/vt+vt/vt (n=16)**	**wt (n=86)**	**wt/vt (n=9)**	**vt/vt (n=5)**	**wt/vt+vt/vt (n=14)**
Total As	28.2 ± 27.7	17.5 ± 12.6	11.2 ± 10.5	16.4 ± 12.2	21.9 ± 20.0	36.4 ± 59.5	24.1 ± 17.3	31.7 ± 47.0
As^{III}	2.8 ± 2.7	1.5 ± 0.9	1.3 ± 0.9	1.5 ± 0.9	22.9 ± 3.5	4.8 ± 6.4	2.5 ± 2.5	3.9 ± 5.2
As^{V}	**1.4 ± 2.5** *b*	1.7 ± 1.4	1.0 ± 0.0	**1.6 ± 1.3** *b*	**0.8 ± 1.1** *b*	2.0 ± 2.1	2.1 ± 3.4	**2.0 ± 2.6** *b*
MMA^{V}	5.0 ± 4.9	3.1 ± 2.1	4.0 ± 3.4	3.3 ± 2.1	3.6 ± 3.8	7.2 ± 11.8	4.0 ± 2.8	5.9 ± 9.0
DMA^{V}	21.7 ± 21.8	13.6 ± 8.7	10.9 ± 0.5	13.2 ± 8.1	16.2 ± 13.3	27.9 ± 42.6	15.3 ± 10.1	22.6 ± 32.7
iAs	3.2 ± 3.3	2.0 ± 1.9	1.2 ± 1.6	1.8 ± 1.8	3.0 ± 4.0	5.7 ± 8.4	4.6 ± 4.4	5.3 ± 6.9
First Methylation	1.8 ± 2.4	1.8 ± 1.3	2.1 ± 0.1 *b*	**1.8 ± 1.2** *b*	3.3 ± 7.5	1.1 ± 0.8	1.1 ± 0.3 *b*	**1.1 ± 0.6** *b*
Second Methylation	5.9 ± 4.6	4.9 ± 2.5	4.1 ± 3.3	4.8 ± 2.5	6.3 ± 6.0	5.8 ± 2.2	4.0 ± 1.3	5.1 ± 2.0

a,b p<0.05: Mann-Whitney.

a comparison intra-group (wt/wt *vs* wt/vt and vt/vt and wt/vt+vt/vt).

b comparison between cases and controls (wt/wt *vs* wt/wt; wt/vt *vs* wt/vt; vt/vt *vs* vt/vt and wt/vt+vt/vt *vs* wt/vt+vt/vt).

Alelos: wt/wt = wild type (homozygous), wt/vt= heterozygous.

Table 7.

Adjusted association between lung cancer with style life, arsenic levels and with the studied polymorphisms.

	β	IC (95%)	P
Smoking index	0.007	0.004 - 0.019	0.000
Total As	0.000	−0.001 - 0.003	0.465
As3MT	0.114	−0.042 - 0.271	0.151
MRP1	0.164	0.011 - 0.316	0.035
P-gp	0.075	−0.115 - 0.266	0.436

Adjusted by: schooling, cooking with wood, smoking and type of drinking water.

Table 8.

Adjusted odds ratios for lung cancer in the studied subjects.

	OR	IC (95%)	P
Smoking index	1.08	1.04 - 1.12	0.000
Total As	1.00	0.99 - 0.01	1.016
As3MT	1.64	0.82 - 3.24	0.155
MRP1	2.08	1.05 - 4.12	0.034
P-gp	1.39	0.60 - 3.22	0.435

Adjusted by: schooling, cooking with wood, smoking and type of drinking water.