ORIGINAL ARTICLE

Urinary chromium is associated with changes in leukocyte miRNA expression in obese subjects

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BACKGROUND/OBJECTIVES: Epidemiological studies suggest a link between chromium (Cr) status and cardiovascular disease. Increased urinary excretion of Cr was reported in subjects with diabetes compared with non-diabetic controls and those with non-diabetic insulin resistance. Epigenetic alterations have been linked to the presence of Cr, and microRNA (miRNA) expression has been implicated in the pathogenesis of metabolic diseases and cardiovascular diseases (CVDs). We investigated the association between Cr excretion and miRNA expression in leukocytes from obese subjects. We also examined the relationship between altered miRNA expression and selected clinical parameters to further investigate mechanisms linking Cr to metabolic diseases and CVDs.

SUBJECTS/METHODS: We analyzed urinary Cr in 90 Italian subjects using inductively coupled plasma-mass spectrometry. Peripheral blood miRNA levels were screened with TaqMan Low-Density Array Human MicroRNA A. Cr level-associated expression of miRNAs was detected with multivariate regression analyses, and the top 10 candidate miRNAs were selected for validation. We also used multivariate regression analyses to assess possible associations between validated miRNAs and glycated hemoglobin (A1c) and blood pressure (BP). The validated miRNAs were further investigated by functional analysis with Ingenuity Pathway Analysis software.

RESULTS: Urinary Cr levels (mean: 0.35 μg/l; s.d. = 0.24) ranged from 0.05 to 1.27 μg/l. In the screening phase, 43 miRNAs were negatively associated with Cr. Of the top 10 miRNAs selected for validation, nine (miR-451, miR-301, miR-15b, miR-21, miR-26a, miR-362-3p, miR-182, miR-183 and miR-486-3p) were downregulated in association with Cr (P-false discovery rate (FDR) < 0.10). miR-451 expression was associated with A1c (β = -0.06; P = 0.0416), whereas miR-486-3p expression was associated both with diastolic (β = 2.1; P = 0.004) and systolic BP (β = 3.3; P = 0.003).

CONCLUSIONS: These results indicate that miR-451 and miR-486-3p are involved in the link between Cr levels and metabolic diseases and CVDs.

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INTRODUCTION

Chromium (Cr) is the sixth most abundant element in the earth’s crust and seawater, existing in several oxidation states, principally metallic, trivalent and hexavalent Cr. Trivalent Cr, an essential trace element for humans, is a critical cofactor for insulin¹ and has a well-documented role in maintaining normal glucose tolerance.² Chromium (Cr) is the sixth most abundant element in the earth crust and seawater, existing in several oxidation states, primarily metallic, trivalent and hexavalent Cr. Trivalent Cr, an essential trace element for humans, is a critical cofactor for insulin¹ and has a well-documented role in maintaining normal glucose tolerance.² Dietary Cr is available in drinking water and various foods, including meats, whole-grains, some fruits, vegetables and spices.⁵ The recommended daily dietary Cr intake for healthy adults is 24–35 μg from age 14 to 50 years and 20–30 μg after 50 years of age.⁶ Once adsorbed into the blood, Cr is widely distributed, but is most concentrated in the kidneys, muscles and liver.⁷ Cr effects are dependent upon the form and amount of supplemental Cr Excretion of absorbed Cr occurs primarily via urine.⁸ Higher than normal urinary Cr excretion has been reported for diabetics⁹ and non-diabetic insulin-resistant patients.¹⁰,¹¹ Epidemiological studies have suggested that Cr status may be linked with cardiovascular diseases (CVDs).¹²,¹³

Epigenetic alterations, such as DNA methylation, posttranslational histone modifications and alterations of small non-coding RNAs, are linked to the presence of Cr in organisms¹⁴ and regulate homeostatic and inducible gene expression.¹⁵ MicroRNAs (miRNAs) are non-coding, small, single-stranded RNAs approximately 21–25 nucleotides long that have important regulatory roles in gene expression. They downregulate expression of target genes at the posttranscriptional level by binding to the 3’ untranslated regions of target mRNAs.¹⁶ Aberrant miRNA expression has been implicated in several metabolic processes, including energy and lipid metabolism, which have been studied in the context of diabetes,¹⁷ insulin resistance¹⁸ and CVD.¹⁹ Obese, defined as a body mass index (BMI) of 30 kg/m² or greater, is strongly associated with onset of type 2 diabetes,²⁰,²¹ insulin resistance²² and CVD.²³ Therefore, obese individuals may represent a suitable population to investigate the epigenetic mechanisms linking Cr levels with metabolic diseases and CVDs. The glycated hemoglobin (A1c) value is an integrated measure of mean glucose levels over time and is considered the ‘gold standard’ for monitoring metabolic control in patients with insulin resistance and diabetes.²⁴ Elevated blood pressure (BP) is a well-established risk factor for CVD; an increase in systolic BP of 1 mm Hg has been estimated to raise the risk of death due to CVD by 2–4%.²⁵

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This study aimed to verify a possible association between urinary Cr levels and miRNA expression in leukocytes from obese subjects. In addition, we sought to examine the relationship between altered miRNA expression and two clinical parameters, A1c and diastolic/systolic BP, to further investigate mechanisms linking Cr to metabolic diseases and CVDs.

MATERIALS AND METHODS

Study design

Ninety subjects were recruited from the ‘Center for Obesity and Weight Control’ at ‘Ospedale Maggiore Policlinico, Fondazione IRCCS Ca’ Granda’ in Milan, Italy. The study subjects were a subgroup of the SPHERE (‘Susceptibility to Particle Health Effects, miRNA, and Exosomes’) project, funded by ERC-2011-StG (282413) to examine molecular mechanisms underlying the effects of particulate matter and metal exposure on health outcomes. Detailed information on this population has been reported previously. Each study participant provided written informed consent in compliance with the ethics committee of the ‘Ospedale Maggiore Policlinico’ (approval number 1425) and donated 15 ml of blood and 50 ml of urine. A lifestyle questionnaire collecting information on sociodemographic data, residence, education, smoking history, eating habits, personal and family medical history, medication used in the last year, and physical activity was administered to each participant. The lifestyle questionnaire is a short version of the questionnaire used in the EAGLE study (environment and genetics in lung cancer etiology, a multidisciplinary study carried out in Europe). The questionnaire is checked for completeness at the time of data collection in order to ensure high-quality data.

Urine sampling and Cr analysis

Urine samples were collected in polyethylene tubes and delivered to the laboratory at room temperature within 2 h after collection. 5 ml aliquots were stored at –20 °C until analysis, which occurred within 12 months after collection. Before analysis, samples were thawed at room temperature, then mixed and heated for 30 min at 37 °C to dissolve the sediment. One milliliter of urine was diluted with 4 ml aqueous nitric acid, 0.05% v/v (from ultrapure nitric acid 67%, Merck, Darmstadt, Germany), containing the stock solution IV-ICP-MS-71D with standards (Inorganic Ventures, Christiansburg, VA, USA). All solutions were prepared using Milli-Q ultrapure water (conductivity 0.056 μS/cm) (Merck). Samples were subjected to inductively coupled plasma-mass spectrometry (ICP-MS) with an ICP-MS X Series II machine (Thermo Scientific, Rodano, Italy) using the kinetic energy discrimination mode to reduce interferences, operating with H2 in He (8% quenching and 2% auxiliary gas) with a flow of 0.6 l/min and a cooling gas flow of 13 l/min. Ions m/z 52 and 89 for Cr and yttrium as the analytic and internal standards, respectively, were acquired with a dwell time of 10 ms.

miRNA expression data analysis

Real-time PCR permitted quantitative measurement, expressed as related cycle threshold (Ct) of miRNA expression. Ct is the number of PCR cycles at which the efficiency of the real-time PCR reaction is maximal for each miRNA target. In the screening phase, the starting point was a data set containing, for each subject, the Ct values of 377 miRNAs and an AmpScore value, which is a quality index, ranging from 0 to 2, which indicates amplification curve quality in the linear phase. We applied an automatic miRNA selection, setting the values of miRNAs with Ct > 33 and/or with AmpScore < 1, and/or with missing Ct, equal to the detection limit of 35 (Figure 1). To reduce background noise, we excluded miRNAs not expressed in at least 90% of samples (Ct = 35), producing a final data set of 123 miRNAs. The mean Ct of the three endogenous small RNA controls was used as an internal control to normalize the amount of individual miRNA in each sample. Relative quantification of miRNA expression was determined by the ΔΔCt method in which expression levels of each normalized miRNA were calculated relative to a calibrator sample that consisted of a pool of 90 samples; it was used as the 1 x sample (or 100%), and the relative value of each miRNA was expressed as an n-fold difference from the calibrator (Figure 1).
The same preliminary data cleaning and normalization methods were applied to the subsets of miRNAs selected for validation.

Statistical analysis

Descriptive statistics were performed on all variables. Normally distributed data were reported as means ± s.d. or frequencies, as appropriate. We used linear regression models to test the association between urinary Cr and miRNA expression in both the screening and validation phases. MiRNA expression, quantified by relative quantification, was log transformed to achieve a normal distribution.

Multivariate regression analyses, adjusted for age, sex, BMI, smoking habits and granulocyte percent, were used to evaluate changes in miRNA expression in association with urinary Cr level on the recruitment day. Owing to the high number of comparisons made, we took into account a correction for multiple comparisons based on the false discovery rate (FDR) control. A threshold of 0.10 was applied on FDR P-value significance (P-FDR) to identify the top miRNAs among the 377 screened. The same analyses were repeated in the validation phase on the set of miRNAs selected for confirmation. Multivariate regression models, adjusted for sex, age, BMI, granulocyte percentage and smoking habits, were also applied to assess the possible association between confirmed miRNAs and two selected clinical parameters: A1c and diastolic/systolic BP. Logarithmic transformations were applied, when appropriate, to achieve normal distributions of outcome variables in regression models. We conducted a residual fit analysis to confirm the model fit. All analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA).

Prediction of miRNA targets and pathway analysis

Data from the top 10 miRNA were submitted to the IPA Core for Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA) to investigate the signaling and metabolic pathways, molecular networks and biological processes associated with them. Putative miRNA–mRNA relationships were identified with the IPA microRNA Target Filter, based on a knowledge base of prediction with high-confidence and experimentally observed relationships from TargetScan, TarBase, miRecords and Ingenuity Knowledge Base. The resulting target gene lists were analyzed to identify diseases and biological functions associated with miRNA gene sets.

We filtered the results for CVDs, nutritional diseases and metabolic diseases, and the resulting target gene list, in addition to nine miRNAs, was analyzed with IPA core analysis. Functional analysis was carried out to identify canonical pathways and molecular networks that have been associated with the miRNA target gene set. The IPA database considers gene–phenotype associations, molecular interactions, regulatory events and chemical knowledge to provide a global molecular network. Related networks were constructed algorithmically based on connectivity. The statistical significance of each biological function was calculated using Fisher's exact test with alpha set to 0.05.

RESULTS

Characteristics of study participants and urinary Cr assessment

The main characteristics of the study participants are presented in Table 1. Briefly, a majority of the participants were women, and a wide age range (21–75 years) was represented. All subjects had a BMI > 25 kg/m², and 62.2% were obese (BMI > 30 kg/m²). We observed a wide range of urinary Cr levels (0.05–1.27 μg/l). In addition, 43.1% of subjects were insulin resistant, and 8.3% of subjects were diabetic. Hypertension was observed in 33.9% of subjects.

miRNA expression screening and validation in association with urinary Cr

After data cleaning, 123 miRNAs were analyzed, according to the criteria described in the Materials and methods section. In the screening phase, we identified 43 miRNAs that were negatively associated with urinary Cr levels (P < 0.05, P-FDR < 0.10). Table 2 shows the percentage change of miRNA expression in terms of relative quantification per 1% increment in urinary Cr. Ten candidate miRNAs were chosen for validation, on the basis of their significant P-value and P-FDR. Consistent with the screening data, nine miRNAs (miR-451, miR-301, miR-15b, miR-21, miR-26a, miR-362-3p, miR-182, miR-183 and miR-486-3p) were negatively associated with urinary Cr level, with a P-FDR < 0.10 (Table 3). The mean coefficient of variability of this study was 0.89%.

miRNA associations with clinical parameters

miR-451 expression associated negatively with A1c (Δ% = –0.06%; P = 0.0416) (Figure 2a), whereas miR-486-3p expression associated positively with both diastolic (Δ% = 2.11%; P = 0.004; Figure 2b) and systolic BP (Δ% = 3.34%; P = 0.003; Figure 2c). None of the
other miRNAs were associated with the examined clinical parameters (data not shown).

Target gene identification and functional analysis
We identified 4473 mRNAs that were experimentally observed or highly predicted targets. When the data were filtered for metabolic and signaling pathways only, we found 1570 mRNA targets that mapped to several molecular networks. To investigate if the mRNAs targeted by our nine validated urinary Cr-associated miRNAs (Table 3) were potentially involved in biological processes connected to obesity and related diseases, the list was filtered for cardiovascular, nutritional and metabolic diseases, resulting in 764 genes (Supplementary Table 1). The number of targeted mRNAs for each miRNA was: miR-451 = 16, miR-301a = 47, miR-26a = 147, miR-362-3p = 62, miR-182 = 185, miR-183 = 80 and 486-3p = 111.

To understand the biological functions of the miRNA targets, we performed IPA’s core analysis, and the top canonical pathways were molecular mechanisms of cancer (P = 7.96 × 10−6), axonal guidance signaling (P = 2.25 × 10−4), protein kinase A signaling (P = 8.46 × 10−4), role of NFAT in cardiac hypertrophy (P = 1.13 × 10−2) and phosphatase and tensin homolog (PTEN) signaling (P = 2.64 × 10−2). The top two networks were enriched for nervous system development and function, metabolic category and for cardiovascular system development and function, respectively.

| Characteristic | NR (proportion) | Mean ± s.d. |
|---------------|-----------------|-------------|
| Sex           |                 |             |
| Men           | 15 (16.7%)      |             |
| Women         | 75 (83.3%)      |             |
| Age, (years)  |                 |             |
| 51.6 ± 11.9   |                 |             |
| BMI, (kg/m²)  |                 |             |
| 32.9 ± 5.7    |                 |             |
| Overweight (25 ≤ BMI < 30) | 34 (37.8%) |             |
| Class I obesity (30 ≤ BMI < 35) | 28 (31.1%) |             |
| Class II obesity (35 ≤ BMI < 40) | 19 (21.1%) |             |
| Class III obesity (BMI ≥ 40) | 9 (10.0%) |             |
| Smoke         |                 |             |
| Never smoked  | 48 (53.2%)      |             |
| Former smoker | 26 (28.9%)      |             |
| Current smoker| 16 (17.8%)      |             |
| Blood count   |                 |             |
| White blood cells (%) | 7.0 ± 2.0 |             |
| Granulocytes (%) | 61.3 ± 7.6 |             |
| Monocytes (%)  | 8.4 ± 5.2       |             |
| Lymphocytes (%) | 30.4 ± 7.6     |             |
| Red blood cells (%) | 4.8 ± 0.5 |             |
| A1C (mmol/mol) | 41.6 ± 8.4     |             |
| Chromium, (µg/l) | 0.35 ± 0.24 |             |
| Diastolic pressure (mmHg) | 79.2 ± 10.2 |         |
| Systolic pressure (mmHg) | 124.8 ± 16.5 |        |

Table 1. Study cohort characteristics (N=90)

Abbreviations: A1C, glycated hemoglobin; BMI, body mass index; NR, number. Continuous variable are expressed as mean ± s.d. Discrete variables are expressed as counts (%).

DISCUSSION
To further investigate the mechanisms linking Cr to metabolic and cardiovascular disorders, we analyzed miRNA expression in leukocytes from obese subjects in association with urinary Cr, and examined how altered miRNA expression related to A1C and diastolic/systolic BP. Obese individuals were chosen for this study because of their strong susceptibility to diabetes, insulin resistance and CVD. We identified and validated a distinct signature of nine miRNAs in peripheral blood that associated negatively with urinary Cr and among these only miR-451 was associated with A1C, suggesting a role for miR-451 in the development of diabetes. Meanwhile, miR-486-3p was associated with systolic and diastolic BP, suggesting it may be a factor in CVD risk.

The two most widespread oxidation states of Cr, Cr⁶⁺ and Cr⁷⁺, have different mobility and bioavailability, resulting in differential
Diabetes may have altered Cr metabolism compared with non-diabetic controls, and re-established the method in elemental analysis, because of its unique measurement of urine Cr concentration by ICP-MS is a well-validated tool in coronary artery disease\(^{38,39}\) and MI.\(^{40,41}\) miR-15b, by modulating apoptosis and angiogenesis, is involved in mitogen-activated protein kinase signaling and suppresses mesangial cells, which may differ in sequence by only a single base.\(^{53}\) miRNAs perform key roles in critical biological processes that affect an underlying cardiac hypertrophy. PI3K is a known regulator of skeletal muscle hypertrophy and atrophy.\(^{44}\) The increase of miR-486-3p in the plasma of acute MI patients may reflect an underlying cardiac hypertrophy.\(^{46}\)

Our results revealed a Cr-related downregulation in miR-26a and miR-21 expression; some authors have also found that miR-26a was downregulated in the sera of patients with acute MI.\(^{46}\) MiR-21 exhibits increased expression in several forms of CVD and is sensitive to high-concentration glucose treatment in macrophages, providing resistance to apoptosis induced by high glucose concentrations via programmed cell death 4 (PDCD4) regulation.\(^{48}\) MiR-182 and miR-183 constitute a well-known miRNA cluster, located on chromosome 7, which is involved in insulin signaling and energy metabolism. Xu and Wong\(^{49}\) provided experimental evidence indicating that this miRNA cluster, miR-183-96-182, targets Irs1, Rasa1 and Grb2, the products of which are all in the insulin signaling pathway. In our obese subjects, two components of this cluster (miR-182 and miR-183) were significantly related to urinary Cr, suggesting their correlation with the insulin pathway.

We also found that miR-301a and miR-362 were negatively associated with urinary Cr, and our IPA core analysis indicated that the PTEN signaling pathway was the top canonical pathway related to expression of these miRNAs. MiR-301a and miR-362 are involved in the NF-kB (nuclear factor kappa light-chain enhancer of activated B cells) and insulin pathways. MiR-301a, which modulates Kv4.2 (potassium voltage-gated channel subfamily D2) by binding to its 3'-UTR, is downregulated in the hearts of diabetes patients, indicating the direct association of mir-301a with diabetes.\(^{50}\) MiR-301a regulates IL-6-induced insulin resistance by direct regulation of PTEN expression.\(^{51}\)

Elevation of miR-451 in red blood cells may affect its quantification in leukocytes, but a strength of our study was the use of the PAXgene Blood System, which allows complete red blood cells lysis and loss before miRNA isolation. Another strength of this study was the use of a non-targeted, exploratory approach to select candidate miRNAs. We chose to use a real-time PCR approach for miRNA expression analysis because it provides sufficiently high precision and sensitivity to detect closely related miRNAs, which may differ in sequence by only a single base.\(^{53}\) Furthermore, to limit false positives in the detection of differentially expressed miRNAs, we used restrictive statistical significance cutoffs.

Our population is composed essentially of female gender, but a recent study\(^{54}\) showed that sex seemed to have a weak effect on the miRNA levels. With the aim of taking into account the different distribution of males and females, and different obesity levels in our population we decided to use multivariate models to select candidate miRNAs. We chose to use a real-time PCR approach for miRNA expression analysis because it provides sufficiently high precision and sensitivity to detect closely related miRNAs, which may differ in sequence by only a single base.\(^{53}\)

In our study population, the mean urinary Cr level was higher than that found in the general Italian population\(^{55}\) and was higher
than the mean concentration recommended by the Institute of Medicine of the National Research Council (0.22 μg/l). This finding could be explained by the high percentage of people with insulin resistance in the study. We used one of the most robust commercially available ICP-MS instruments, which provided many advantages, including elemental specificity and high measurement sensitivity over a wide linear dynamic range.56 One limitation of our study was the lack of Cr speciation; to achieve Cr speciation, ICP-MS analysis would have to be coupled with high performance liquid chromatography.57 In addition, from the initial list of 43 miRNAs, we chose to validate only 10 based on their P-FDR values; it is possible that we may have excluded some relevant miRNAs involved in Cr metabolism. We did adjust for potential confounding variables in our analysis. Moreover, the relatively small sample size may have limited our ability to detect significant effects, and larger studies are needed to confirm our findings.

CONCLUSION

Several circulating miRNAs are considered emerging blood biomarkers for many diseases because they are stable molecules, tissue specific, evolutionarily conserved and easy to detect. Changes in miR-451 and miR-486-3p expression, in association with urinary Cr, and their relation with A1c and BP, respectively, are helpful for identifying mechanisms linking Cr with metabolic diseases and CVDs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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