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Interaction of tissue inhibitor of metalloproteinase 3 gene polymorphism, blood cadmium and total urinary arsenic levels on clear cell renal cell carcinoma

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In renal cell carcinoma (RCC), tissue inhibitor of metalloproteinase (TIMP) 3 expression is lost, suggesting that the *TIMP3* gene may function as a tumor suppressor gene. Cadmium (Cd) and arsenic (As) exposure may affect the expression of TIMP3. Here we investigate the association of clear cell RCC with *TIMP3* polymorphisms, and explore whether *TIMP3* polymorphisms modify the relationship between blood Cd or total urinary As levels and clear cell RCC respectively. We recruited 281 clear cell RCC patients and 689 sex- and age-matched controls. The clear cell RCC was diagnosed by pathological evaluation after image-guided biopsy or surgical resection of the renal tumor. Concentrations of blood Cd and lead, and also total urinary As, were measured. We determined *TIMP3* polymorphisms using the Agena Bioscience MassARRAY system. Odds ratio (OR) of clear cell RCC was significantly inversely correlated with *TIMP3* rs9609643 GA/AA genotype, with OR = 0.63 (95% confidence interval, CI, 0.44–0.91). For *TIMP3* rs715572 AA compared to the GG/GA genotype, the OR of clear cell RCC was 1.60 with 95% CI of 1.01–2.56. Individuals with high blood Cd concentrations and the *TIMP3* rs9609643 GG genotype exhibited a higher OR of clear cell RCC than reference groups (OR = 4.48, 95% CI 2.09–9.60). This study presents a novel finding that the GA/AA genotype of *TIMP3* rs9609643 significantly decreased the clear cell RCC risk, and AA genotype of *TIMP3* rs715572 significantly increased the clear cell RCC risk. Furthermore, this study first identified that the *TIMP3* rs9609643 risk genotypes appear to interact with high blood Cd levels to increase the OR of clear cell RCC.

Keywords Tissue inhibitor of metalloproteinases 3, Cadmium, Arsenic, Clear Cell Renal cell carcinoma

Renal cell carcinoma (RCC) originates from renal tubular epithelial cells—it is the most prevalent form of kidney cancer. Clear cell histology accounts for approximately 70–80% of RCC cases, and other histological types include papillary and chromophobe cells[1]. In 2020, there were an estimated 431,288 new renal cancer cases worldwide, with epidemiological data showing that RCC accounts for the majority (90%) histologically[2]. Globally, RCC represents around 2% of cancer incidence and mortality rates, with future increases projected[2]. The age-standardized incidence rate of RCC rose from 3.39/10^6 in 2002 to 5.09/10^6 in 2012 in Taiwan[3]. Potential risk factors for RCC include comorbidities like urolithiasis, hypertension, and diabetes, lifestyle factors such as smoking and obesity, as well as environmental factors[4]. Environmental factors lead (Pb) and cadmium (Cd) in

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the blood, were found to be significantly higher in RCC cases than controls[5]. Our previous study also revealed an association of high total urinary arsenic (As) and high blood Cd levels with the RCC odds ratio (OR)[6]. However, determining the molecular mechanisms that comprise the relationship between RCC and these metals require further investigation.

Tissue inhibitor of metalloproteinases (TIMP) 3 is a member of the TIMP family and serves as an endogenous regulator of matrix metalloproteinases (MMPs)—it is vital to maintaining the surrounding extracellular matrix (ECM). Its broad inhibitory spectrum against MMP activity makes TIMP3 a key player in ECM regulation[7]. Studies examining methylation-associated silencing of TIMP3 have suggested its role as a tumor suppressor in various cancers. Loss of TIMP3 expression has been linked to the development of tumorigenesis[8,9]. Notably, nearly all cases of clear cell RCC exhibit loss of TIMP3 expression[10]. Recent research revealed that FK506-binding protein 51 binds to TIMP3, facilitating its connection to the Beclin1 complex. This interaction enhances the autophagic degradation of TIMP3 and significantly promotes the invasion and migration of RCC[11]. These findings underscore the important role of TIMP3 in RCC progression.

Inorganic As has been shown to enhance the expression of MMP genes, which play a role in degrading the ECM[12], thereby influencing As-related cancers. In rat hepatocytes, Cd was found to decrease expressions of TIMP2 and TIMP3, which are positively regulated by ten-eleven translocation methylcytosine dioxygenase 1 (TET1), which participates in DNA demethylation[13]. Moreover, urinary levels of As and Cd exhibited a positive and significant correlation with TIMP1, a marker associated with renal damage[14]. Cell experiments have revealed that cells exposed to As developed pathological fibrosis features, potentially attributed to the upregulation of fibrosis-associated signaling molecules such as TIMP3 and MMP2[15]. However, these studies provide inconsistent results concerning the relationship between As and Cd exposure and TIMP3.

The gene *TIMP3* is found on chromosome 22q12.1-q13.2[16], and is considered a putative tumor suppressor gene. It belongs to a family of four members known as TIMPs, with TIMP3 a 24-kDa secreted glycoprotein inhibiting proteolytic activity of MMPs, and exhibiting anti-metastatic and anti-tumorigenic properties[17]. The functional significance of most reported TIMP3 variants remains unclear, although these single nucleotide polymorphisms (SNPs) can be related to the clinical outcome of cancer. For instance, one study found a significant association between the *TIMP3* rs9619311 TC/CC genotype and muscle-invasive urothelial cell carcinoma in non-smokers, whereas *TIMP3* rs11547635 was not linked to this cancer[18]. One study indicated that the *TIMP3* rs715572 AG/AA genotype was significantly associated with colorectal cancer relative to the GG genotype[19]. In terms of breast cancer, women of *TIMP3* rs9609643 AA genotype exhibited a significantly lower risk compared to the GG genotype, while women with the *TIMP3* rs8136803 TT genotype showed a significantly greater likelihood of breast cancer compared to the GG genotype[20]. Furthermore, the *TIMP3* rs2234921 G allele showed an increased skin cancer risk when combined with high levels of As exposure, compared to the *TIMP3* rs2234921 A allele combined with low As exposure levels[21]. However, there has been no investigation of the role of *TIMP3* polymorphism in RCC. Therefore, we aimed to examine the association of *TIMP3* polymorphism with clear cell RCC. Additionally, we explored whether *TIMP3* polymorphism could modify the respective relationships of blood Cd and total urinary As concentration with clear cell RCC.

Materials and methods

Study subjects

The study was a case–control. The inclusion criteria for participants were those with pathological diagnosis of RCC or clinical imaging consistent with RCC, and those aged 20–80 years old. People without RCC or other cancers served as controls. Exclusion conditions included those with incomplete specimens and data or those who refused to sign the consent form. This study recruited 380 patients who were diagnosed with RCC through pathological confirmation. The control group consisted of 689 individuals who were matched with the age and sex of RCC patients, and they did not have RCC or any other malignancy[6]. Around 70% of the RCC patients had grade II or III tumors. Specifically, there were 281 cases of clear cell carcinoma, 27 of papillary carcinoma, 29 of chromophobe carcinoma, 6 of sarcoma, and 7 classified as "other." Information regarding the cell type of 33 cases was not available. Because TIMP3 is related to angiogenesis mediated by vascular endothelial growth factor (VEGF) and VEGFR-2, it is also a key marker of clear cell renal cancer[10,22]. Therefore, this study mainly used 281 people with clear cell RCC as cases. Prior to their participation, all study participants provided informed consent in writing, including questionnaire interviews and specimen collection. The Research Ethics Committee of National Taiwan University Hospital approved the research protocol (approval no. 201705091RINC, date 2021-07-02), and it was conducted according to the Declaration of Helsinki by the World Medical Association.

Questionnaire interview and biospecimen collection

The methodology for questionnaire interviews and information collection was previously detailed[6]. Blood samples were collected using an EDTA vacuum syringe in a volume of 5–8 mL and then processed with the separation of blood cells to measure Pb and Cd levels. Buffy coats were isolated for DNA extraction and subsequent *TIMP3* genotyping. Additionally, spot urine samples were collected and kept at − 20 °C prior to analyzing As species.

Measurements of blood Cd and Pb and urinary As levels

Quantification of blood Cd and Pb levels was conducted using inductively coupled plasma mass spectrometry (Agilent Technologies, Santa Clara, CA, USA)[23]. The separation of arsenite, arsenate, monomethylarsinic acid, and dimethylarsinic acid was achieved through high-performance liquid chromatography (Merck Hitachi, Tokyo, Japan), followed by hydride generation coupled with an atomic absorption spectrometer (PerkinElmer, Waltham, MA, USA) to measure the concentration of the four substances[24]. The summed concentrations of these four urinary As species was termed total urinary As concentration, and this was then divided by urine creatinine

concentration, thus adjusting for hydration status[25]. This analytical method is not affected by arsenobetaine, arsenocholine or arsenosugar found in seafood. Our previous study showed that the frequency of fish, shellfish, and seaweed consumption was not significantly associated with the concentration of inorganic arsenic and its methylated species[26]. For measurement methods, standard reference materials, recovery rate, detection limits, and reliability for metal measurements, see Supplementary Table S1.

Determining TIMP3 gene polymorphisms

To extract genomic DNA, the samples were subjected to digestion with proteinase K, followed by phenol–chloroform extraction. We selected six common *TIMP3* SNPs from the Han Chinese population using Beijing HapMap data. The allelic exchange, global mutant allele frequency (MAF), and the gene's polymorphism location of *TIMP3* are shown in Table 1. The somatic mutation was performed by Agena MassARRAY platform with iPLEX reagent chemistry (Agena, San Diego, CA, USA). The specific PCR primer and extension primer sequences of *TIMP3* rs715572, *TIMP3* rs2234921, *TIMP3* rs8136803, *TIMP3* rs9609643, *TIMP3* rs9619311, and *TIMP3* rs11547635 were designed with Assay Designer software package (v.4.0) (Supplementary Table S2). Of genomic DNA sample (10 ng/μl), 1 μl was applied to mutiplex PCR reaction in 5-μl volumes containing 1 unit of Taq polymerase, 500 nmol of each PCR primer mix, and 2.5 mM of each dNTP (Agena, PCR accessory and Enzyme kit). Thermocycling was at 94 °C for 4 min followed by 45 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 1 min, then 72 °C for 3 min. Unincorporated dNTPs were deactivated using 0.3 U of shrimp alkaline phosphatase. The single base extension reaction was using iPLEX Pro enzyme, terminator mix, and extension primer mix, followed by 94 °C for 30 s followed by 40 cycles of 94 °C for 5 s, and five inner cycles of 56 °C for 5 s and 80 °C for 5 s, then 72 °C for 3 min (Agena, iPLEX Pro reagent kit). After the addition of a cation exchange resin to remove residual salt from the reactions, 7 nl of the purified primer extension reaction was loaded onto a matrix pad of a SpectroCHIP (Agena). SpectroCHIPs were analyzed using a MassARRAY Analyzer 4, with the calling by clustering analysis with TYPER 4.0 software. The distribution of all control genotypes obeyed Hardy–Weinberg equilibrium.

Statistical analysis

The Wilcoxon rank sum test was implemented for between-groups comparison of continuous variables. The distribution of categorical variables between the groups and whether the control group *TIMP3* genotypes fitted Hardy–Weinberg equilibrium were determined using Chi-square test. In the control group, continuous independent variables were categorized into three groups, with the first tertile serving as the reference category. Multivariate logistic regression models were used for calculation of OR and corresponding 95% confidence intervals (CIs), and so assess the relationship between clear cell RCC and the categorical independent variables. To examine linear trends in ORs across the strata of categorical independent variables, the categorical variables were treated as continuous variables. Haploview 4.1 software was employed to assess the strength of linkage disequilibrium (LD) by calculating D′ and r^2 of Lewontin 1[27]. Interactions between metal concentrations and *TIMP3* polymorphisms in relation to clear cell RCC were evaluated using the median of metal concentration in the control group as the cutoff point. A logistic regression model with a product term was used to test for multiplicative interactions between the two variables. Additive interactions were evaluated using several measures, including attributable proportion (AP), synergy index, and relative excess risk due to interaction (RERI)[28]. Data analysis was conducted using SAS software (version 9.4; Cary, NC, USA), with two-tailed significance of $p < 0.05$, and marginally significant $0.05 < p < 0.1$.

Results

Comparison of sociodemographic and lifestyle factors between clear cell RCC cases and controls

Table 2 displays a comparison of disease history, sociodemographics, and lifestyle factors between clear cell RCC patients and the non-RCC (controls). Between the groups, there were no significant differences in distributions of age and sex. The clear cell RCC patients exhibited a higher proportion of individuals with illiteracy and primary education level compared to the controls. In contrast to clear cell RCC cases, the control group reported significantly higher rates of occasional or frequent consumption of alcohol, tea, and coffee, but had a lower cumulative pack-year of smoking. The clear cell RCC patients had significantly higher rates of diabetes and hypertension than the controls.

TIMP3	Allelic Exchange	Global MAF	Gene's Polymorphism Location
rs715572	G > A	A = 0.216515	32,838,944
rs2234921	A > G	G = 0.36649	32,801,088
rs8136803	G > A, T	T = 0.078006	32,841,125
rs9609643	G > A	A = 0.053910	32,855,072
rs9619311	T > C	C = 0.320978	32,800,707
rs11547635	C > A, T	T = 0.074334 T = 0.112540	32,857,305

Table 1. The allelic exchange, global mutant allele frequency (MAF), and the gene's polymorphism location of *TIMP3*. The information of all SNP from The Allele Frequency Aggregator (ALFA), rs11547635 also from The Genome Aggregation Database (GnomAD_exome).

Variables	Clear cell RCC cases (*N* = 281)	Controls (*N* = 689)	Age-gender adjusted ORs (95% CI)
Age	58.78 (49.00, 69.00)	59.00 (51.00, 71.00) [a]	0.99 (0.98–1.00)
Gender			
Male	185 (66.84)	442 (64.15) [b]	1.00
Female	96 (34.16)	247 (35.85)	0.93 (0.69–1.124)
Educational level			
Illiterate/elementary school	63 (22.42)	133 (19.36) [b,*]	**1.00**[§,**]
Junior/senior high school	105 (37.37)	231 (33.62)	0.85 (0.57–1.26)
College and above	113 (40.21)	323 (47.02)	**0.60 (0.40–0.91)**[*]
College and above vs. high school and below			**0.67 (0.44–0.91)**[*]
Cumulative cigarette smoking (pack year)	0.00 (0.00, 16.75)	0.00 (0.00, 6.00) [a,+]	**1.01 (1.00–1.02)**[*]
Alcohol consumption			
Never	214 (76.16)	410 (59.51) [b,***]	**1.00**[§,***]
Frequently	53 (18.86)	115 (16.69)	0.77 (0.53–1.13)
Occasional	14 (4.98)	164 (23.80)	**0.15 (0.08–0.26)**[***]
Frequently and Occasional	67 (23.84)	279 (40.49)	**0.40 (0.29–0.56)**[***]
Coffee consumption			
Never	183 (65.36)	322 (46.74) [b,***]	**1.00**[§,***]
Frequently	74 (26.43)	180 (26.12)	**0.70 (0.50–0.97)**[*]
Occasional	23 (8.21)	187 (27.14)	**0.22 (0.14–0.35)**[***]
Frequently and Occasional	97 (34.64)	367 (43.26)	**0.45 (0.34–0.61)**[***]
Tea consumption			
Never	144 (51.43)	229 (33.24) [b,***]	**1.00**[§,***]
Frequently	113 (40.36)	283 (41.07)	**0.61 (0.45–0.83)**[**]
Occasional	23 (8.21)	177 (25.69)	**0.21 (0.13–0.33)**[***]
Frequently and Occasional	136 (48.57)	460 (66.76)	**0.45 (0.34–0.60)**[***]
Diabetes			
No	222 (79.29)	632 (92.13) [b,***]	1.00
Yes	58 (20.71)	54 (7.87)	**3.30 (2.20–4.97)**[***]
Hypertension			
No	150 (53.58)	514 (74.93) [b,***]	1.00
Yes	131 (46.62)	172 (25.07)	**2.93 (2.16–3.97)**[***]

Table 2. Comparison of sociodemographic characteristics, lifestyle, and disease history between clear cell RCC patients and non-RCC controls. Clear cell RCC, Clear cell renal cell carcinoma; Values are expressed as median (first quartile, third quartile) or number (%) of cases and controls. Two participants were missing for educational level; three missing for diabetes and hypertension. There were 19 missing for cumulative cigarette smoking in controls, and four missing for cumulative cigarette smoking in clear cell RCC cases. [a]Wilcoxon rank-sum test. [b]χ^2 test. $^{+}0.05 < p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. [§] trend test,

Polymorphisms and haplotypes of *TIMP3* and clear cell RCC risk

Associations between polymorphisms and haplotypes of *TIMP3* and RCC are given in Table 3. In multivariable adjusted models, clear cell RCC cases had significantly higher odds of having the *TIMP3* rs715572 AA compared to the GG/GA genotype (OR, 1.60; 95% CI, 1.01–2.56). Conversely, clear cell RCC cases had significantly lower odds of having the *TIMP3* rs9609643 GA/AA genotype (OR, 0.63; 95% CI, 0.44–0.91) compared to the GG genotype. There were no significant associations for other *TIMP3* polymorphisms. Haplotype analyses identified two haplotype blocks within the *TIMP3* gene (Supplementary Figure S1): block 1 (*TIMP3* rs9619311 and *TIMP3* rs2234921), and block 2 (*TIMP3* rs9609643 and *TIMP3* rs11547635). Among these, only block 2 exhibited high LD, with a D′ value of 0.97 (Figure S1A), and r^2 values (Figure S1B) indicated the strength of the LD between these two polymorphisms. The A-C and A-T haplotypes in block 2 showed a significant inverse association with clear cell RCC compared to the G-C and G-T haplotypes, with an OR of 0.64 (95% CI, 0.46–0.89).

Metals and clear cell RCC risk

The clear cell RCC cases exhibited significantly higher levels of blood Cd, blood Pb, and total urinary As than controls (Table 4). Following multivariate adjustment, clear cell RCC cases with total urinary As levels > 25.04 µg/L had 1.87-fold (95% CI, 1.11–3.16) significantly higher odds of clear cell RCC than those with total urinary As levels ≤ 11.89 µg/L. Similarly, clear cell RCC cases demonstrated 5.41-fold (95% CI, 3.06–9.55) increased odds of blood Cd levels of > 1.64 µg/L compared to ≤ 0.92 µg/L after adjusting for multiple variables. However, blood Pb levels and clear cell RCC showed no significant association.

TIMP3 genotypes	Clear cell RCC Cases	Controls	Age-gender adjusted ORs (95% CI)	Multivariate adjusted ORs (95% CI)[a]
rs11547635 C > T				
CC	112 (40.14)	298 (43.44)	1.00	1.00
CT	133 (47.67)	318 (46.36)	1.09 (0.81–1.47)	1.08 (0.78–1.50)
TT	34 (12.19)	70 (10.20)	1.29 (0.81–2.05)	1.22 (0.73–2.03)
CT/TT	167 (59.86)	388 (56.56)	1.12 (0.85–1.50)	1.13 (0.84–1.53)
rs2234921 A > G				
AA	227 (91.07)	570 (83.21)	1.00	1.00
AG	51 (18.21)	113 (16.50)	1.14 (0.79–1.65)	1.16 (0.77–1.73)
GG	2 (0.71)	2 (0.29)	2.61 (0.36–18.76)	2.68 (0.33–21.79)
AG/GG	53 (18.92)	115 (16.79)	1.17 (0.81–1.67)	1.18 (0.80–1.76)
rs715572 G > A				
GG	119 (42.65)	286 (42.06)	1.00	1.00
GA	123 (44.09)	326 (47.94)	0.91 (0.68–1.23)	0.92 (0.66–1.27)
AA	37 (13.26)	68 (10.00)	1.31 (0.83–2.06)	1.53 (0.93–2.52)$^{+}$
AA vs. GG/GA	37/242	68/612	1.37 (0.89–2.11)	**1.60 (1.01–2.56)***
rs9609643 G > A				
GG	223 (79.64)	488 (71.76)	1.00	1.00
GA	56 (20.00)	173 (25.44)	0.71 (0.51–1.00)$^{+}$	**0.68 (0.47–0.99)***
AA	1 (0.36)	19 (2.79)	**0.11 (0.02–0.86)***	0.13 (0.02–1.04)$^{+}$
GA/AA	57 (20.36)	192 (28.24)	**0.65 (0.47–0.92)***	**0.63 (0.44–0.91)***
rs9619311 T > C				
TT	227 (81.36)	570 (83.33)	1.00	1.00
TC	50 (17.92)	112 (16.37)	1.13 (0.78–1.63)	1.17 (0.78–1.75)
CC	2 (0.72)	2 (0.29)	2.59 (0.36–18.62)	2.65 (0.33–21.67)
TC/CC	52 (18.64)	114 (16.67)	1.15 (0.80–1.66)	1.20 (0.81–1.78)
rs8136803 G > T				
GG	254 (90.39)	625 (90.71)	1.00	1.00
GT	26 (9.25)	63 (9.14)	1.02 (0.63–1.65)	0.97 (0.58–1.62)
TT	1 (0.36)	1 (0.15)	2.67 (0.17–43.05)	4.07 (0.20–84.48)
GT/TT	27 (9.61)	64 (9.29)	1.05 (0.65–1.68)	1.00 (0.60–1.67)
Haplotypes of *TIMP3*	Clear cell RCC Cases	Controls	Age-gender adjusted ORs (95% CI)	Multivariate adjusted ORs (95% CI)[a]
Block 2 (rs9609643 and rs11547635)				
G-C	297 (53.61)	685 (51.20)	1.00	1.00
G-T	199 (35.92)	445 (33.26)	1.02 (0.82–1.27)	0.99 (0.78–1.26)
A-C and A-T G-C and G-T A-C and A-T	58 (10.47) 496 (89.53) 58 (10.47)	208 (15.55) 1130 (84.45) 208 (15.55)	**0.64 (0.47–0.89)**** 1.00 **0.64 (0.47–0.87)****	**0.63 (0.45–0.90)**** 1.00 **0.64 (0.46–0.89)****

Table 3. Associations between polymorphisms and haplotype of *TIMP3* and clear cell RCC. Clear cell RCC, Clear cell renal cell carcinoma; *TIMP3*, tissue inhibitor of metalloproteinase 3; OR, odds ratio; CI, confidence interval. Five missing for *TIMP3*rs11547635 and *TIMP3*rs2234921; seven missing for *TIMP3*rs9619311; 10 missing for *TIMP3*rs9609643; and 11 missing for *TIMP3*rs715572. [a]Adjusted for age; sex; educational level; cumulative cigarette smoking; alcohol, coffee, and tea consumption; and disease histories of diabetes and hypertension. $^{+}0.05 < p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$.

Interaction of metals and genotype or haplotype of *TIMP3* on clear cell RCC risk

Table 5 presents the combined effects on clear cell RCC of *TIMP3* polymorphisms and total urinary As levels, or blood Cd concentrations. After adjusting for multivariable, clear cell RCC cases showed 2.27-fold (95% CI, 1.23–4.18) increased odds of having total urinary As levels > 11.22 μg/L and possessing *TIMP3* rs9609643 GG genotype compared to urinary total As levels ≤ 11.22 μg/L and *TIMP3* rs9609643 GA/AA genotype. Furthermore, ORs progressively increased from having no risk factors (i.e., low total urinary As levels and *TIMP3* rs9609643 GA/AA genotype) to having one risk factor (i.e., high total urinary As levels or *TIMP3* rs9609643 GG genotype), and to having two risk factors (i.e., high total urinary As levels and *TIMP3* rs9609643 GG genotype). There were similar associations when assessing the combination of blood Cd levels and *TIMP3* rs9609643 genotype, and that of blood Cd or total urinary As levels and *TIMP3* rs715572 genotype on clear cell RCC. The *TIMP3* rs9609643 GG genotype tended to interact multiplicatively with high blood Cd level to increase the OR of clear cell RCC. However, all additive interactions were nonsignificant. The effects of the combination of *TIMP3* haplotype and total urinary As, as well as blood Pb concentrations, on clear cell RCC, are presented in Supplementary Table

Variables	Clear cell RCC cases ($N=281$)	Controls ($N=689$)	Age-sex adjusted ORs (95% CI)	Multivariate ORs (95% CI)
Total urinary arsenic concentration (μg/L)	**12.28 (5.53, 22.52)**[a, ***]	**17.22 (9.44, 29.79)**[a, ***]		
≤ 11.89	136 (48.40)	230 (33.38)	**1.00**[b, §, **]	**1.00**[c, §, *]
11.89–25.04	86 (30.60)	230 (33.38)	**1.47 (1.00–2.17)**[*]	1.51 (0.99–2.30)[+]
> 25.04	59 (21.00)	229 (33.24)	**1.85 (1.15–2.96)**[*]	**1.87 (1.11–3.16)**[*]
Total urinary arsenic concentration (μg/g creatinine)	**17.08 (10.61, 26.87)**[a, **]	**14.92 (9.76, 23.96)**[a, **]		
≤ 11.22	78 (27.76)	230 (33.38)	**1.00**[§, **]	**1.00**[d, §, **]
11.22–20.14	89 (31.67)	230 (33.38)	1.20 (0.84–1.72)	1.39 (0.94–2.05)
> 20.14	114 (44.57)	229 (33.24)	**1.65 (1.16–2.37)**[**]	**1.72 (1.16–2.56)**[**]
Blood lead level (μg/dL)	44.73 (33.62, 62.92)	46.32 (30.78, 67.14)		
≤ 36.14	49 (32.67)	224 (33.38)	1.00	1.00[d]
36.14–58.54	57 (38.00)	224 (33.38)	1.17 (0.77–1.79)	**1.63 (1.02–2.60)**[*]
> 58.54	44 (29.33)	223 (33.24)	0.89 (0.57–1.40)	1.16 (0.70–1.90)
Blood cadmium level (μg/L)	**1.85 (1.16, 2.96)**[***]	**1.26 (0.78, 2.00)**[***]		
≤ 0.92	24 (16.00)	229 (34.13)	1.00[§, ***]	**1.00**[d, §, ***]
0.92–1.64	40 (26.67)	220 (32.79)	**1.84 (1.07–3.17)**[*]	**2.36 (1.31–4.24)**[**]
> 1.64	86 (57.33)	222 (33.08)	**3.99 (2.43–6.57)**[***]	**5.41 (3.06–9.55)**[***]

Table 4. The association between total urinary arsenic level, blood lead and cadmium levels and clear cell RCC. Values expressed as median (first quartile, third quartile) or number (percent). One hundred and thirty one participants were missing for blood cadmium and lead concentration. [a]Wilcoxon rank-sum test. [b]Adjusted for age, sex, and urinary creatinine level. [c]Adjusted for age; sex; creatinine level; cumulative cigarette smoking; educational level; alcohol, coffee, and tea consumption; analgesic usage; diabetes; and hypertension. [d]Adjusted for age; sex; cumulative cigarette smoking; educational level; alcohol, coffee, and tea consumption; analgesic usage; diabetes; and hypertension. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. [§] Trend test.

S3. When examining the combined effects of blood Cd or total urinary As concentrations with the risky *TIMP3* haplotype in block 2, the clear cell RCC OR significantly rose with dose as the number of risk factors increased, i.e., high blood Cd levels, high total urinary As concentrations, or presence of *TIMP3* haplotype block 2 (G-C and G-T). However, all additive and multiplicative interactions were nonsignificant.

Negative and positive predictors for clear cell RCC

Finally, positive and negative predictors for clear cell RCC were determined using stepwise logistic regression analysis (Table 6). Age, sex, frequent and occasional consumption of alcohol and tea were identified as significant negative predictors for clear cell RCC. However, elevated blood Cd levels, a history of diabetes and hypertension, and the presence of the *TIMP3* rs715572 AA genotype were significant positive predictors for clear cell RCC.

Discussion

There was a significant negative association between the *TIMP3* rs9609643 GA/AA compared to the GG genotype, and the haplotype of *TIMP3* (rs9609643 and rs11547635) A-C and A-T compared to G-C and G-T, with clear cell RCC. We also found a significant positive association between *TIMP3* rs715572 (AA vs. GG/GA genotype) and clear cell RCC. Furthermore, high blood Cd levels showed a tendency to interact multiplicatively with *TIMP3* rs9609643 (GG vs. GA/AA) increasing the OR for clear cell RCC.

There have been few investigations of *TIMP3* rs9609643. A study found a 60% lower likelihood of breast cancer for women with the *TIMP3* rs9609643 AA than the GG genotype (OR, 0.4; 95% CI, 0.2–1.0)[20]. There have also been few studies on the relationship between *TIMP3* rs715572 and cancer. Compared to the GG genotype, *TIMP3* rs715572 AG/AA was associated with increased risk of colorectal cancer[19]. *TIMP3* rs715572 (CC vs. CT/TT) was associated with survival of adenocarcinomas of the gastroesophageal junction[29]. In this study, we discovered a significant association between the *TIMP3* rs9609643 GA/AA genotype and a reduced risk of clear cell RCC. In addition, *TIMP3* rs715572 AA was significantly related with increased OR of clear cell RCC. To our knowledge, these findings are relatively novel in the clear cell RCC field.

Functional polymorphisms in *TIMP3* genes have been implicated in the modulation of activity, thereby influencing the clinical characteristics of prostate cancer[30]. Patients carrying the *TIMP3* rs9619311 TC + CC polymorphism showed an increased risk of prostate cancer recurrence, but *TIMP3* rs11547635 was not associated with this cancer[30]. A study suggested that the *TIMP3* rs2234921 GG genotype was marginally associated with a higher risk of skin cancer than AA/AG genotype[21]. The *TIMP3* rs8136803 TT genotype showed a significantly greater risk of breast cancer compared to the GG genotype[20]. However, in our study, there was no association of *TIMP3* rs2234921, *TIMP3* rs8136803, *TIMP3* rs11547635, or *TIMP3* rs9619311 with clear cell RCC. These findings indicated inconsistent results from current studies concerning the relationship of *TIMP3* rs2234921, *TIMP3* rs8136803, *TIMP3* rs11547635, and *TIMP3* rs9619311 with cancer.

Metals	TIMP3 genotypes	Clear cell RCC cases/ controls	Age-gender adjusted ORs (95% CI)	Multivariate adjusted ORs (95% CI)[a]
Total urinary arsenic (μg/L)	rs9609643 G > A			
≤ 11.22	GA/AA	29/59	**1.00**[a,&,***]	**1.00**[b,&,***]
≤ 11.22	GG	103/160	1.43 (0.85–2.43)	1.30 (0.73–2.32)
> 11.22	GA/AA	28/133	1.35 (0.70–2.63)	1.25 (0.61–2.58)
> 11.22	GG	120/328	**2.39 (1.36–4.20)**[**]	**2.27 (1.23–4.18)**[**]
		RERI	0.61 (-0.32–1.53)	0.71 (-0.22–1.64)
		AP	0.25 (-0.13–0.64)	0.32 (-0.10–0.73)
		Synergy index	1.77 (0.50–6.30)	2.29 (0.33–15.82)
		$p_{interaction}$	0.169	0.202
Blood cadmium (μg/L)	rs9609643 G > A			
≤ 1.26	GA/AA	10/97	**1.00**[c,&,***]	**1.00**[c,&,***]
≤ 1.26	GG	35/239	1.39 (0.66–2.93)	1.51 (0.69–3.30)
> 1.26	GA/AA	26/90	**2.92 (1.33–6.41)**[**]	**3.44 (1.45–8.16)**[**]
> 1.26	GG	79/237	**3.32 (1.65–6.69)**[***]	**4.48 (2.09–9.60)**[***]
		RERI	0.010 (-1.73–1.74)	0.53 (-1.66–2.71)
		AP	0.003 (-0.52–0.53)	0.12 (-0.37–0.61)
		Synergy index	1.00 (0.47–2.13)	1.18 (0.56–2.49)
		$p_{interaction}$	**0.050**	0.080
Total urinary arsenic concentration (μg/g creatinine)	rs9609643 G > A			
≤ 14.92	GA/AA	29/82	**1.00**[a,&,***]	**1.00**[b,&,***]
≤ 14.92	GG	90/256	0.98 (0.60–1.60)	1.11 (0.66–1.88)
> 14.92	GA/AA	28/110	0.77 (0.43–1.41)	0.93 (0.48–1.77)
> 14.92	GG	113/232	**1.75 (1.08–2.83)**[*]	**1.99 (1.18–3.34)**[**]
		RERI	0.99 (0.44–1.55)	0.95 (0.25–1.65)
		AP	0.57 (0.22–0.91)	0.48 (0.11–0.85)
		Synergy index	-3.09 (-)	28.67 (-)
		$p_{interaction}$	0.284	0.561
Blood cadmium (μg/L)	rs715572 G > A			
≤ 1.26	GG/GA	38/303	**1.00**[c,&,***]	**1.00**[c,&,***]
≤ 1.26	AA	7/33	1.74 (0.72–4.22)	2.50 (0.97–6.44)[+]
> 1.26	GG/GA	87/293	**2.48 (1.63–3.77)**[***]	**3.16 (1.96–5.08)**[***]
> 1.26	AA	18/33	**4.57 (2.33–8.94)**[***]	**6.41 (3.01–13.67)**[***]
		RERI	1.35 (-1.71–4.40)	1.75 (-2.96–6.46)
		AP	0.30 (-0.24–0.83)	0.27 (-0.31–0.86)
		Synergy index	1.61 (0.579–4.52)	1.448 (0.55–3.97)
		$p_{interaction}$	0.103	0.122
Total urinary arsenic (μg/L)	rs715572 G > A			
≤ 11.22	GG/GA	112/191	**1.00**[a,&,*]	**1.00**[b,&,**]
≤ 11.22	AA	20/30	0.99 (0.52–1.887)	1.05 (0.51–2.13)[+]
> 11.22	GG/GA	130/421	**1.55 (1.06–2.28)**[*]	**1.56 (1.02–2.39)**[*]
> 11.22	AA	17/38	**2.06 (1.03–4.11)**[*]	**2.70 (1.29–5.64)**[**]
		RERI	0.52 (-0.92–1.96)	1.08 (-0.84–3.01)
		AP	0.25 (-0.32–0.83)	0.40 (-0.09–0.89)
		Synergy index	1.96 (0.31–12.45)	2.78 (0.46–16.72)
		$p_{interaction}$	0.853	0.978
Total urinary arsenic concentration (μg/g creatinine)	rs715572 G > A			
≤ 14.92	GG/GA	98/301	**1.00**[c,&,**]	**1.00**[c,&,**]
≤ 14.92	AA	20/38	1.61 (0.89–2.91)	1.91 (1.0–3.66)[+]
> 14.92	GG/GA	144/311	**1.54 (1.13–2.11)**[**]	**1.66 (1.17–2.34)**[**]
Continued				

Metals	TIMP3 genotypes	Clear cell RCC cases/ controls	Age-gender adjusted ORs (95% CI)	Multivariate adjusted ORs (95% CI)[a]
> 14.92	AA	17/30	**1.90 (1.00–3.61)***	**2.38 (1.17–4.84)***
		RERI	-0.26 (-1.76–1.23)	-0.19 (-2.18–1.80)
		AP	-0.14 (-0.99–0.71)	-0.08 (-0.96–0.80)
		Synergy index	0.77 (0.17–3.49)	0.88 (0.22–3.47)
		$p_{interaction}$	0.172	0.299

Table 5. Combined effects of *TIMP3* polymorphisms and total urinary arsenic and blood lead concentrations on clear cell RCC. Abbreviations: TIMP3, tissue inhibitor of metalloproteinase 3; Clear cell RCC, Clear cell renal cell carcinoma; OR, odds ratio; CI, confidence interval; RERI, relative excess risk due to interaction; AP, attributable proportion. [&]Tested for linear trend. $^{+}0.05 < p < 0.1$, $^{**}p < 0.01$, $^{***}p < 0.001$. [a]Adjusted for age, sex, and urinary creatinine. [b]Adjusted for age, sex, urinary creatinine, educational level, analgesic usage, disease histories of diabetes and hypertension, and alcohol, coffee, and tea consumption. [c]Adjusted for age, sex, educational level, analgesic usage, disease histories of diabetes and hypertension, and alcohol, coffee, and tea consumption.

Variables	OR (95% CI) for clear cell RCC
Age (1 age increment)	**0.98 (0.97–0.99)***
Sex (Female vs. male)	**0.62 (0.40–0.95)***
Concentrations of blood cadmium (1 μg/L increment)	**1.19 (1.08–1.32)*****
Alcohol consumption (Frequently and Occasional vs. never)	**0.47 (0.30–0.74)****
Tea consumption (Frequently and Occasional vs. never)	**0.61 (0.41–0.90)***
TIMP3 rs715572 (AA vs. GG/GA)	**2.12 (1.23–3.66)****
Diabetes	**2.93 (1.74–4.94)*****
Hypertension	**2.96 (1.97–4.44)*****

Table 6. Stepwise multiple logistic regression analysis. TIMP3, tissue inhibitor of metalloproteinase 3; clear cell RCC, clear cell renal cell carcinoma. Variables included age; sex; concentrations of blood lead and cadmium and total urinary arsenic; alcohol, tea and coffee consumption; educational level; *TIMP3* rs9609643 genotype; *TIMP3* rs715572 genotype; and disease histories of diabetes and hypertension in the stepwise multiple logistic regression model. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

However, *TIMP3* rs9609643 appeared to alter the association between blood Cd concentration and clear cell RCC. Individuals carrying *TIMP3* rs9609643 GG genotypes had an increased risk of clear cell RCC associated with high blood Cd concentrations compared to those with *TIMP3* rs9609643 GA/AA genotypes and low blood Cd concentrations in this study. This is likely the first study to examine the interaction of *TIMP3* genetic polymorphisms with environmental pollutants, specifically Cd. These findings suggest that genetic variation in the *TIMP3* promoter region may contribute to the development of Cd-induced clear cell RCC. It is possible that Cd induces oxidative stress, leading to the production of oxidative stress-sensitive metallothionein 2A, which in turn triggers the activity of TET1 (DNA demethylation) along with apolipoprotein E. Moreover, Cd has been shown to decrease the expression of TIMP2 and TIMP3, which are positively regulated by TET1[13], while TIMP3 expression can be reduced in cancer tissues in comparison with normal controls[31]. Additionally, the *TIMP3* rs9609643 GG genotype may potentially alter protein expression by affecting transcription factor binding sites, thus disrupting the balance between TIMP3 and MMPs, impacting ECM remodeling[32] and increasing the risk of clear cell RCC. However, experimental confirmation of these findings is warranted in future studies. To our knowledge, no other studies have examined the association of this polymorphism with clear cell RCC, and it is not known whether this SNP is functional. Therefore, it would be valuable to study TIMP3 function, which would further understand of the mechanisms of *TIMP3* genotypes in clear cell RCC.

This study has several limitations. First, it is should be noted that this is a case–control study, and so the temporal relationship of environmental factors with clear cell RCC is difficult to clarify. Secondly, the assessment of total urinary As and blood Cd concentrations was based on a single sample. The reliability of these measurements relies on the assumption of a stable lifestyle and metabolism for all patients during the sample collection period. Thirdly, the study had a small sample size, which potentially limits generalizability of the results. Therefore, further validation using an increased sample size is necessary to ensure more robust and meaningful interpretations of the results. Despite these limitations, the study's findings offer valuable insights into factors that may affect Cd-related clear cell RCC.

Conclusions

This study represents the first investigation to identify significant associations between the *TIMP3* rs9609643 GA/AA and *TIMP3* rs715572 AA genotype and clear cell RCC. Additionally, our observational study provides

novel evidence indicating that the risk genotype of *TIMP3* rs9609643 appears to modify the relationship between environmental factors (specifically blood Cd) and clear cell RCC.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization and study design, YMH and CYH; writing—original draft, CYH; writing—review and editing, YMH; statistical analysis, YLH; material preparation, data collection, and analysis, MCC, CYW, HSS, YCL, and YSP; supervision, YMH.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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