DNA methylation of metallothionein genes is associated with the clinical features of renal cell carcinoma

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Abstract. Metallothioneins are low-weight cysteine-rich proteins responsible for metal ion homeostasis in a cell and, thus, capable of regulating cell proliferation and differentiation. Deregulation of metallothionein genes has been reported in various human tumors. However, their role in renal cell carcinoma (RCC) has been poorly investigated. In the present study, we aimed to evaluate the importance of promoter DNA methylation of selected metallothionein genes for RCC. Based on the initial analysis of kidney renal clear cell carcinoma dataset from The Cancer Genome Atlas, genes *MT1E*, *MT1F*, *MT1G* and *MT1M* were selected for qualitative methylation analysis in 30 tumors (including 10 multifocal cases), 10 pericancerous, and 30 non-cancerous renal tissues (NRT). Methylation of *MT1E* and *MT1M* was tumor-specific (P=0.0056 and P=0.0486, respectively) and showed moderate interfocal variation in paired tumor foci. Methylated promoter status of the two genes was associated with larger tumor size (P=0.0110 and P=0.0156, respectively). Furthermore, aberrant *MT1E* methylation was more frequent in tumors having necrotic zones (P=0.0449) or characterized with higher differentiation grade (P=0.0144), while *MT1M* was more commonly methylated in tumors with higher Fuhrman grade (P=0.0272). Only unmethylated *MT1F* promoter status was observed in all analyzed samples. Gene expression analysis (51 RCC and 9 NRT) revealed *MT1G* downregulation in tumors (P<0.0001), while lower *MT1E* expression levels were associated with the promoter methylation (P=0.0077). In clear cell RCC, *MT1E*, *MT1G* and *MT1M* expression was higher than that noted in other histological tumor subtypes (all P<0.0500). In addition, some associations were observed between metabolic syndrome-related clinical parameters and promoter methylation or gene expression. In conclusion, the present study revealed the potential role of *MT1E* and *MT1M* promoter methylation in RCC development.

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Introduction

Metallothioneins are a family of low molecular weight (6-7 kDa), highly conserved, cysteine-rich non-enzymatic cytosolic proteins which play a vital role in metal ion homeostasis. In humans, the majority of metallothionein genes (16 of 19) are located in a cluster on chromosome 16q13, of which 12 are protein-coding (e.g. *MT1A*, *MT1E* and *MT1F*) and the rest are pseudogenes (e.g. *MT1CP*, *MT1L* and *MT1P1*). The main biological roles of metallothioneins in cells are directly related to their ability to bind metal ions. Changes in metallothionein expression alter their major cellular functions, i.e. buffering and delivering zinc and copper, which are critical for proliferating, differentiating and apoptotic cells (1). Due to their affinity for cadmium, lead, or mercury, metallothioneins protect cells from heavy metal toxicity (2,3). They also function as antioxidants against DNA damage caused by free radicals (4).

Metallothioneins have a significant role in cancer development. Gene expression changes have been associated with tumor growth, metastasis and angiogenesis (5). Earlier research has indicated that metallothioneins contribute to the development of resistance to drugs or radiotherapy (6-8); however, recent research has shown that they can also suppress cardiotoxicity induced by anticancer agents (9). Various studies also revealed that metallothionein expression is not universal for different cancer types. Specifically, metallothionein genes are frequently downregulated in liver cancer, squamous cell lung carcinoma, or prostate tumors, which could be associated with promoter DNA methylation (10-12). In contrast, increased metallothionein expression has been reported in melanoma, ovarian or breast tumors (13-15), while investigations of some other tumors produced contradictory results.

Renal cell carcinoma (RCC) comprises ~2-3% of all non-cutaneous cancers and is the most fatal type of urologic malignancies with high mortality rates in Europe. Lithuania has the third highest RCC incidence rate and is in the first place according to mortality worldwide (16). A variety of histologically distinct tumors falls under the RCC definition,

with clear cell RCC (ccRCC) being the most common subtype. Other commonly detected tumors are papillary (pRCC) and chromophobe RCC (chRCC). As clinical outcomes are closely related to tumor stage, grade and other parameters, diagnostic methods for accurate cancer characterization, as well as early detection, are critically important. At present, ~50% of sporadic RCC cases are incidentally detected in asymptomatic patients during examination for other diseases (17). Despite the use of highly sensitive methods, such as computed tomography scan and magnetic resonance imaging, RCC is often detected at already advanced stages when treatment options become limited (18). The lack of diagnostic clinical tests draws attention to genetic and epigenetic features as potential biomarkers of RCC. Novel molecular biomarkers could potentially improve early RCC diagnostics and advise the most beneficial treatment.

In the present study, we investigated aberrant DNA methylation of several protein-coding metallothionein genes aiming to elucidate their importance in renal carcinogenesis. DNA methylation status was compared with clinical-pathological patient characteristics, as well as with gene expression levels. The Cancer Genome Atlas (TCGA) dataset of kidney renal cell carcinoma (KIRC) was used for the screening step. This study led to the identification of the potential importance of *MT1E* and *MT1M* genes in RCC development.

Materials and methods

Patients and samples. In total, 54 patients [22 males and 32 females with the mean age of 63 (41-85) and 67 (27-85), respectively] diagnosed with RCC, who underwent full or partial nephrectomy at the National Cancer Institute (Vilnius, Lithuania) between July 2013 and January 2016, were involved in the study. The study cohort mainly consisted of ccRCC and various cases of other histological subtypes (Table I). Tissues were sampled and grades were assigned to tumors by an expert pathologist. The Fuhrman grade describes adverse morphological characteristics of cell nuclei, whereas the differentiation grade, defined according to the WHO recommendations, is based on tissue histology in general. In total, 54 tumors, 10 paired pericancerous renal tissues (PRT; at a distance of 1-2 cm from the tumor margin), and 33 paired non-cancerous renal tissues (NRT; morphologically normal tissue at >2 cm from the tumor margin and ≤1 cm from the surgical margin if partial nephrectomy was performed) were included in the study (Table I). For 10 patients, two tumor foci were available for the molecular analysis. Approval to conduct biomedical research (no. 158200-13-620-192) was obtained from the Lithuanian Bioethics Committee (Vilnius, Lithuania) before initiating the study, and all patients gave informed consent for participation.

Nucleic acid extraction. Renal tissue samples (~60 mg) were mechanically homogenized with cryoPREP™ CP02 Impactor using tissueTUBEs TT1 (Covaris, Inc., Woburn, MA, USA). For the isolation of genomic DNA, up to 30 mg of tissue powder was treated with proteinase K (Thermo Scientific™; Thermo Fisher Scientific, Vilnius, Lithuania) in 0.5 ml of lysis buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.5% Tween-20; all from Carl Roth GmbH, Co., KG, Karlsruhe, Germany) for up to 18 h at 55˚C and DNA was extracted according to the standard phenol-chloroform purification and ethanol precipitation.

The total RNA was extracted using mirVana™ miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (12). Briefly, ~10 mg of homogenized tissue were treated with 0.5 ml of lysis/binding buffer and 50 μl of miRNA homogenate additive for 10 min in an ice-water bath. The total RNA was extracted with 0.5 ml of acid-phenol:chloroform and purified using the supplied filter cartridges.

Concentration and purity of extracted nucleic acids were evaluated spectrophotometrically with NanoDrop 2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The integrity of randomly selected DNA samples and all RNA samples was analyzed in 1-1.5% agarose gels prepared with 1X TAE buffer (Thermo Fisher Scientific, Inc.) and only intact samples were used for the molecular analysis.

DNA methylation analysis. Bisulfite treatment was applied to 400 ng of extracted DNA using EZ DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocol, except that the initial incubation of samples was performed at 42˚C for 15 min. Methylation-specific PCR (MSP) was used for the qualitative promoter methylation analysis of genes *MT1E*, *MT1G, MT1F* and *MT1M*. Primers specific for methylated or unmethylated DNA (Table II) were designed with Methyl Primer Express Software v1.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.) or selected from the previous publication (12). One microliter of bisulfite-modified DNA was added to 24 μl of MSP mix containing 1.25 U AmpliTaq Gold DNA Polymerase, 1X Gold PCR buffer, 1 μl of 360 GC Enhancer, 2.5 mM $MgCl_2$ (all from Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.4 mM of each dNTP (Thermo Fisher Scientific, Inc.) and 0.5 μM of each primer (Metabion, Martinsried, Germany). Thermocycling conditions were optimized prior to the study and included 35-38 cycles with the primer annealing step at 56-58˚C (Table II). Methylation-positive, methylation-negative, and non-template controls were routinely included. Amplification products were analyzed in 3% agarose gels with 1X TAE buffer and visualized under UV light after ethidium bromide staining (Carl Roth GmbH, Co., KG).

Gene expression analysis. For cDNA synthesis, 250 ng of extracted RNA was reverse transcribed (RT) using High-Capacity cDNA Reverse Transcription kit with RNase Inhibitor following the manufacturer's protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.). Expression levels of genes *MT1E*, *MT1G*, *MT1M* and endogenous control *HPRT1* were evaluated by means of quantitative PCR (RT-qPCR) using TaqMan Gene Expression assays (Hs01582977_gH, Hs01584215_g1, Hs00828387_g1, and Hs02800695_m1, respectively; Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mix (20 μl) consisted of 1X TaqMan Universal Master Mix II no UNG (Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.4X TaqMan Gene Expression assay and 2 μl of RT product. Amplification was performed with Mx3005P™ qPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) in triplicates per gene. Thermocycling consisted of 95˚C for 10 min, followed by 45 cycles of 95˚C

Table I. Clinicopathological characteristics of the RCC study cohorts.

Parameter	All cases (n=54)		Methylation analysis (n=30)		Gene expression analysis (n=51)	
Tissue samples, n						
RCC	54		30		51	
PRT	10		10		0	
NRT	33		30		9	
Histological tumor subtype, n						
ccRCC	41		22		41	
chRCC	3		1		3	
pRCC	1		1		1	
OCT	5		3		3	
Other types	4		3		3	
Tumor size, mean (range), mm	50 (14-130)		54 (14-130)		56 (20-130)	
Pathological tumor stage, n						
≤pT2	31		16		29	
≥pT3	23		14		22	
Fuhrmann grade, n						
F2	19		8		19	
F3	23		16		23	
Unknown	12		6		9	
Differentiation grade[a], n						
≤G2	28		11		28	
G3	19		15		18	
Unknown	7		4		5	
Necrotic zones in tumor, n						
Yes	15		10		14	
No	39		20		37	
Sex	Female, n=32	Male, n=22	Female, n=16	Male, n=14	Female, n=30	Male, n=21
Age at diagnosis, mean (range), years	67 (27-85)	63 (41-85)	66 (27-85)	62 (41-85)	67 (27-85)	63 (41-85)
Waist circumference[b], mean (range), cm	102 (79-135)	100 (78-126)	107 (93-135)	98 (78-122)	101 (79-135)	100 (78-126)
Unknown, n	2	2	1	2	2	2
Raised fasting plasma glucose level[c], n						
Yes	44		6		8	
No	10		24		43	

[a]Differentiation grade was determined according to the recommendations of The World Health Organization. [b]Waist circumference of >102 cm for males and >88 cm for females was considered as high. [c]Fasting glucose level of ≥6.1 mM was considered as high. RCC, renal cell carcinoma; PRT, pericancerous renal tissue; NRT, non-cancerous renal tissue; ccRCC, clear cell RCC; chRCC, chromophobe RCC; pRCC, papillary RCC; OCT, oncocytoma.

for 15 sec and 60˚C for 1 min. Non-template controls were included in each run. Samples with *HPRT1* amplification at cycle ≥35 were considered of low quality and were excluded from the analysis. Data preprocessing was performed using GenEx 6.0.1 software (Multid Analyses AB, Göteborg, Sweden). Relative gene expression values, transformed to a linear scale, were used for statistical analysis.

The Cancer Genome Atlas dataset of renal clear cell carcinoma. For the overview analysis of the metallothionein gene family, the TCGA KIRC dataset was used (19). Global DNA methylation profiling data using Illumina Infinium HumanMethylation450K (HM450) platform and RNA expression data obtained by RNA-seq were utilized in the study. Gene-specific Level 3 datasets were acquired from

Table II. Primers used for methylation-specific PCR (MSP) and amplification conditions.

Gene symbol	Primer ID	Primer sequence (5'-3')	Product size (bp)	Amplicon location from TSS (bp)	PCR cycles	Primer annealing T, (˚C)	(Refs.)
MT1E	M-F	GGATTTCGGGAATATCGC	217	-113/+104	38	56	(12)
	M-R	ACGAAAATCGAACCGAAC					
	U-F	TTTGGATTTTGGGAATATTGT	220	-116/+104			
	U-R	ACAAAAATCAAACCAAACACA					
MT1F	M-F	GTATTCGGAATTTTAAGGGGC	134	-262/-129	35	57	This study
	M-R	CGAACCGTCCCTTTAAAATC					
	U-F	TAGGTATTTGGAATTTTAAGGGGT	139	-265/-127			
	U-R	CACAAACCATCCCTTTAAAATC					
MT1G	M-F	TCGTATACGGGGGGTATAGC	131	-232/-102	37	58	This study
	M-R	GCGATCCCGACCTAAACT					
	U-F	AAGTTGTATATGGGGGGTATAGT	137	-235/-99			
	U-R	CCCACAATCCCAACCTAAACT					
MT1M	M-F	GGATATTGCGTATTATTCGGC	112	-240/-129	38	56	This study
	M-R	ATAAATACCGAACGCACCATC					
	U-F	TTGGGGATATTGTGTATTATTTGGT	116	-244/-129			
	U-R	ATAAATACCAAACACACCATCCC					

TSS, transcription start site; M/U, primer specific for methylated/unmethylated sequence; F/R, forward/reverse primer.

the cBioPortal (http://www.cbiopotal.org) and MethHC (http://methhc.mbc.nctu.edu.tw) data analyses portals in September 2018 (20,21).

Statistical analysis. Statistical analysis was performed using STATISTICA v8.0 (StatSoft Inc., Tulsa, OK, USA). The two-sided Fisher's exact test was used for two-group comparisons of categorical data, while the Mann-Whitney U test was used for continuous data. Heterogeneity index (HI) was calculated to estimate the discordance rate of methylation status of paired tumor foci. Correlations of gene expression levels with quantitative clinicopathological or molecular parameters were evaluated by calculating Spearman's R_S and/or Pearson's R_p correlation coefficients. Receiver Operating Characteristic (ROC) curve analysis was performed and the area under the curve (AUC) was calculated in order to evaluate the clinical utility of the test. Logistic regression analysis was applied for the putative biomarker combination. P-level of <0.0500 was considered significant. Data visualization was developed using GraphPad Prism v5.03 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Metallothionein gene analysis in the TCGA dataset. For the screening step, DNA methylation and gene expression data were extracted for 8 protein-coding metallothionein genes from the TCGA KIRC dataset. In total, data of 520 ccRCC and 209 healthy tissue samples were available for the analysis. Due to the ubiquitous expression of some of the metallothioneins in various tissues and induction by a variety of stimuli (including hormones and growth factors), we focused on *MT1* group genes only. Significant differences in methylation levels between ccRCC and healthy tissues were identified for 5 genes, of which *MT1A*, *MT1E*, *MT1F* and *MT1M* had higher methylation levels in tumors, while *MT1B* was highly, but still differentially methylated in both tissue types (all P<0.0500; Fig. 1). Furthermore, despite the variable range, the methylation intensity of *MT1A*, *MT1E*, *MT1F* and *MT1M* was correlated with downregulated gene expression (all P<0.0500; Fig. 2). Expression of *MT1B* was absent in almost all tumor samples, therefore, it could not be compared with the promoter methylation (data not shown).

DNA methylation analysis of selected metallothionein genes. Based on the TCGA KIRC dataset analysis and with regard to the literature review, four metallothionein genes, *MT1E*, *MT1F*, *MT1G*, and *MT1M*, were selected for the qualitative analysis of promoter DNA methylation. *MT1E*, *MT1G*, and *MT1M* were methylated in ≤43.3% of tumors and less frequently in NRT, but only *MT1E* and *MT1M* showed significant differences (P=0.0056 and P=0.0486, respectively). The three genes were also methylated in PRT indicating the field cancerization phenomenon of the tumor-adjacent area (Fig. 3A). Interfocal variation of methylation status was present in *MT1E* and *MT1M* gene promoters, but no heterogeneity of *MT1G* and *MT1F* was detected (Fig. 3B). Only unmethylated promoter status of *MT1F* was observed in all analyzed tissues.

In our cohort, the sensitivity and specificity of the *MT1E* and *MT1M* gene combination were 53.3 and 83.3%, respectively. The ROC curve analysis of the TCGA KIRC dataset revealed comparable diagnostic values of the two genes

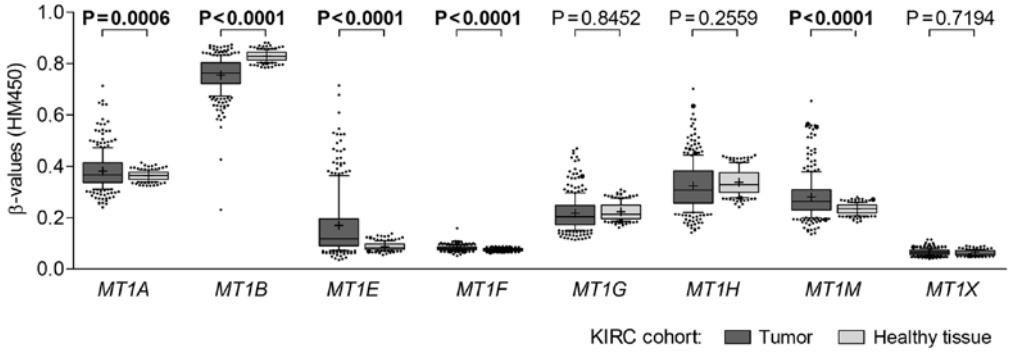

Figure 1. DNA methylation levels of selected protein-coding metallothionein genes in the renal clear cell carcinoma (KIRC) cohort of The Cancer Genome Atlas (TCGA). Level 3 DNA methylation data, obtained using Illumina Human Methylation450K (HM450) platform, was used to generate the plots. The box extends from the 25 to 75th percentiles; the line in the middle of the box is plotted at median; the plus sign depicts the mean; the whiskers represent the 10-90% range; data values outside the range are marked as dots. Significant P-values are in bold.

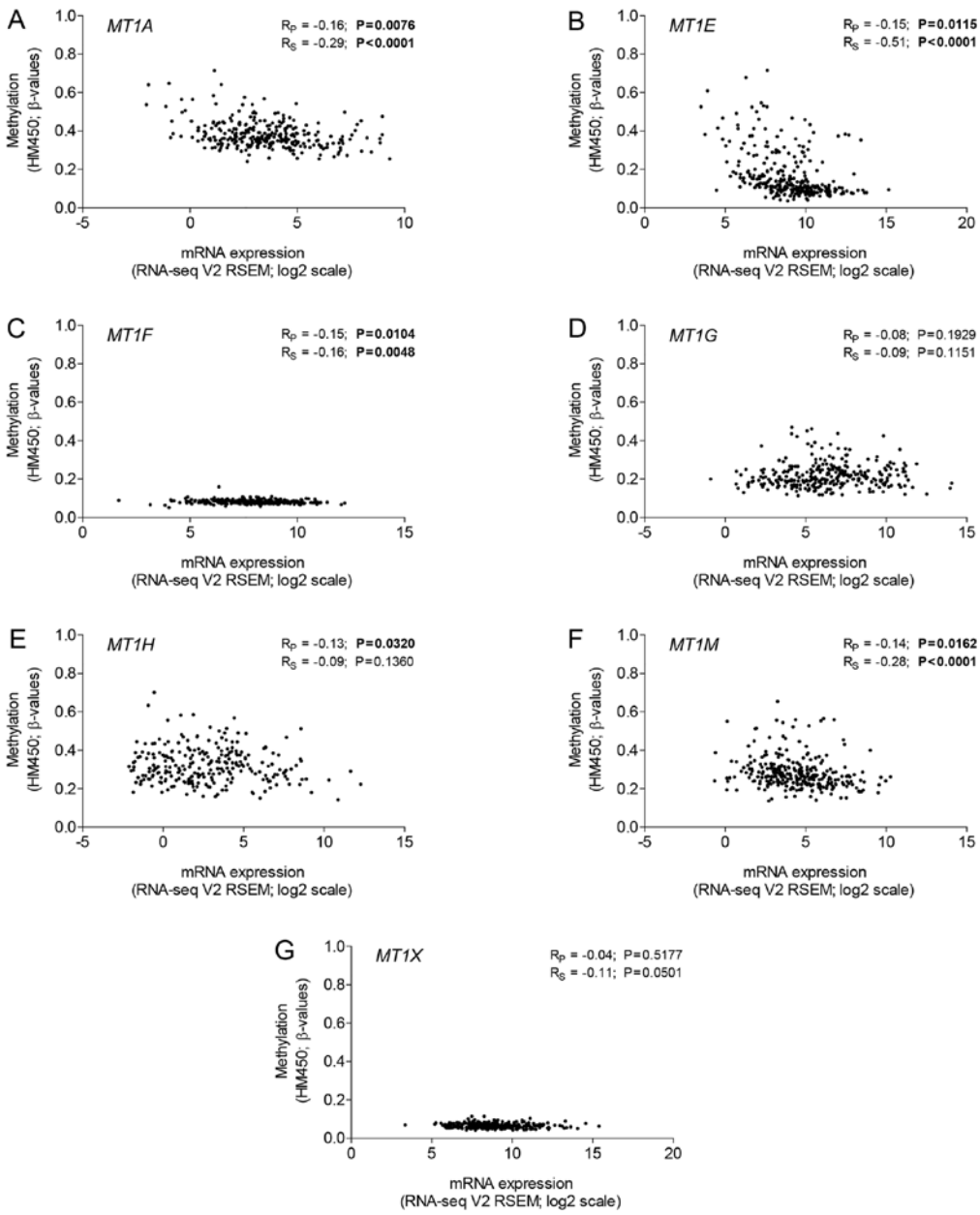

Figure 2. Correlations between promoter methylation and gene expression for (A) *MT1A*, (B) *MT1E*, (C) *MT1F*, (D) *MT1G*, (E) *MT1H*, (F) *MT1M* and (G) *MT1X* in the renal clear cell carcinoma (KIRC) cohort of The Cancer Genome Atlas (TCGA). Level 3 DNA methylation data, obtained using Illumina HumanMethylation450K (HM450) platform, and level 3 KIRC RNA-seq RSEM data were used to generate scatter plots. RNA-seq data is plotted on log2 scale. Pearson's R (R_P) and Spearman's R (R_S) correlation coefficients are provided with respective P-values. Significant P-values are in bold.

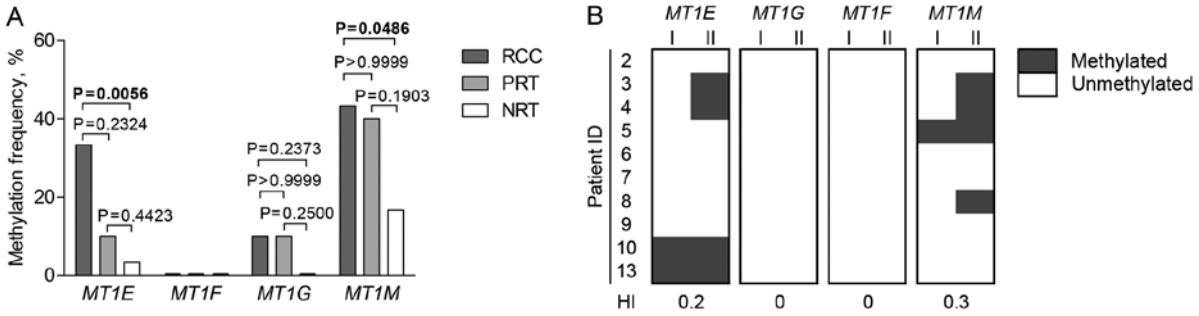

Figure 3. DNA methylation status of genes *MT1E*, *MT1F*, *MT1G* and *MT1M* in renal cancer. (A) Promoter DNA methylation frequencies in tumors (RCC), pericancerous (PRT) and non-cancerous (NRT) renal tissues. (B) Heterogeneity of methylation status in multifocal (I and II foci) carcinoma cases. HI, heterogeneity index. Significant P-values are in bold.

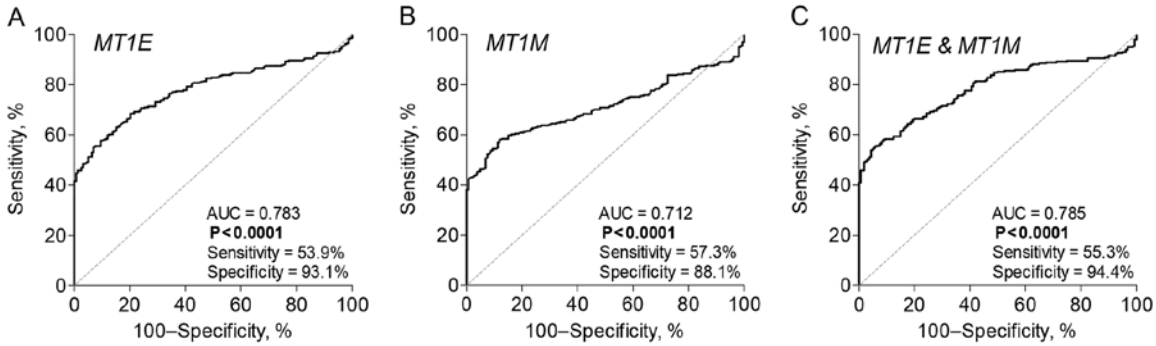

Figure 4. Receiver operating characteristic (ROC) curve analysis of promoter methylation of the selected metallothionein genes in the renal clear cell carcinoma (KIRC) cohort of The Cancer Genome Atlas (TCGA). (A) *MT1E* gene; (B) *MT1M* gene; (C) combination of *MT1E* and *MT1M*. Sensitivity, specificity and area under the curve (AUC) values are provided. Significant P-values are in bold.

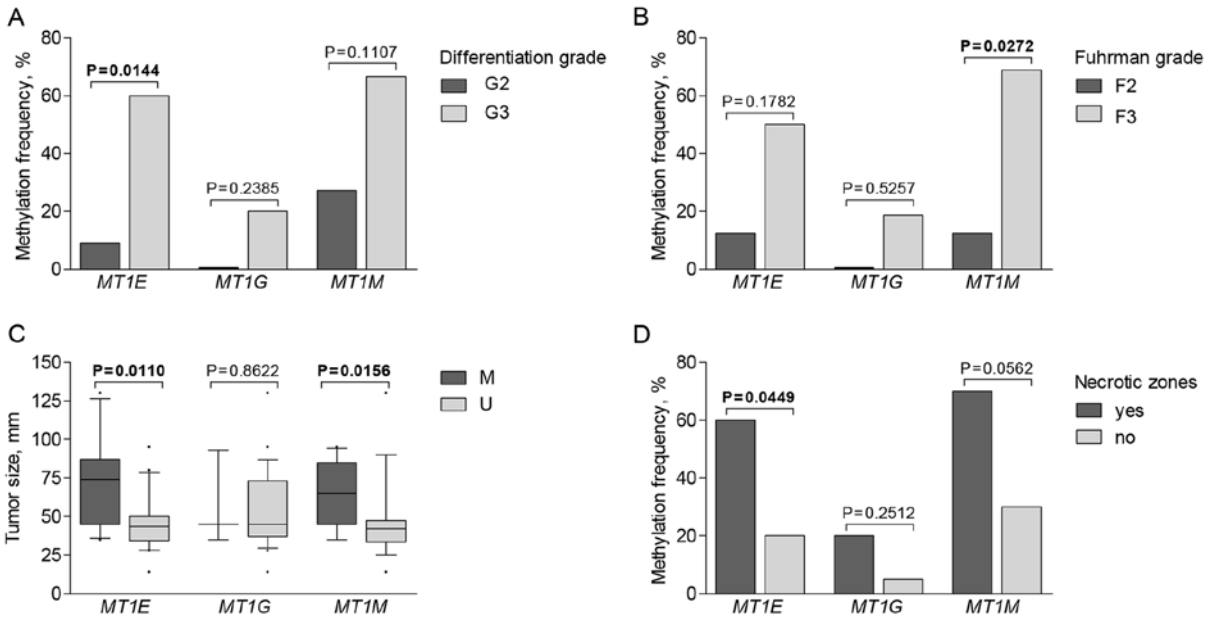

Figure 5. Associations of metallothionein gene promoter methylation in renal tumors (RCC) and clinicopathological patient characteristics. (A) Methylation frequencies according to tumor differentiation grade; (B) methylation frequencies according to Fuhrmann grade; (C) distribution of tumor size according to gene methylation status; (D) methylation frequencies according to the presence of necrotic zones in tumor. The box extends from the 25 to 75th percentiles; the line in the middle of the box is plotted at median; the whiskers represent the 10-90% range; data values outside the range are marked as dots. M/U, methylated/unmethylated gene promoter status. Significant P-values are in bold.

individually or in combination, with the specificity reaching up to 94.4% (Fig. 4).

DNA methylation and clinicopathological parameters. Aberrant promoter methylation of *MT1E*, *MT1G* and *MT1M*

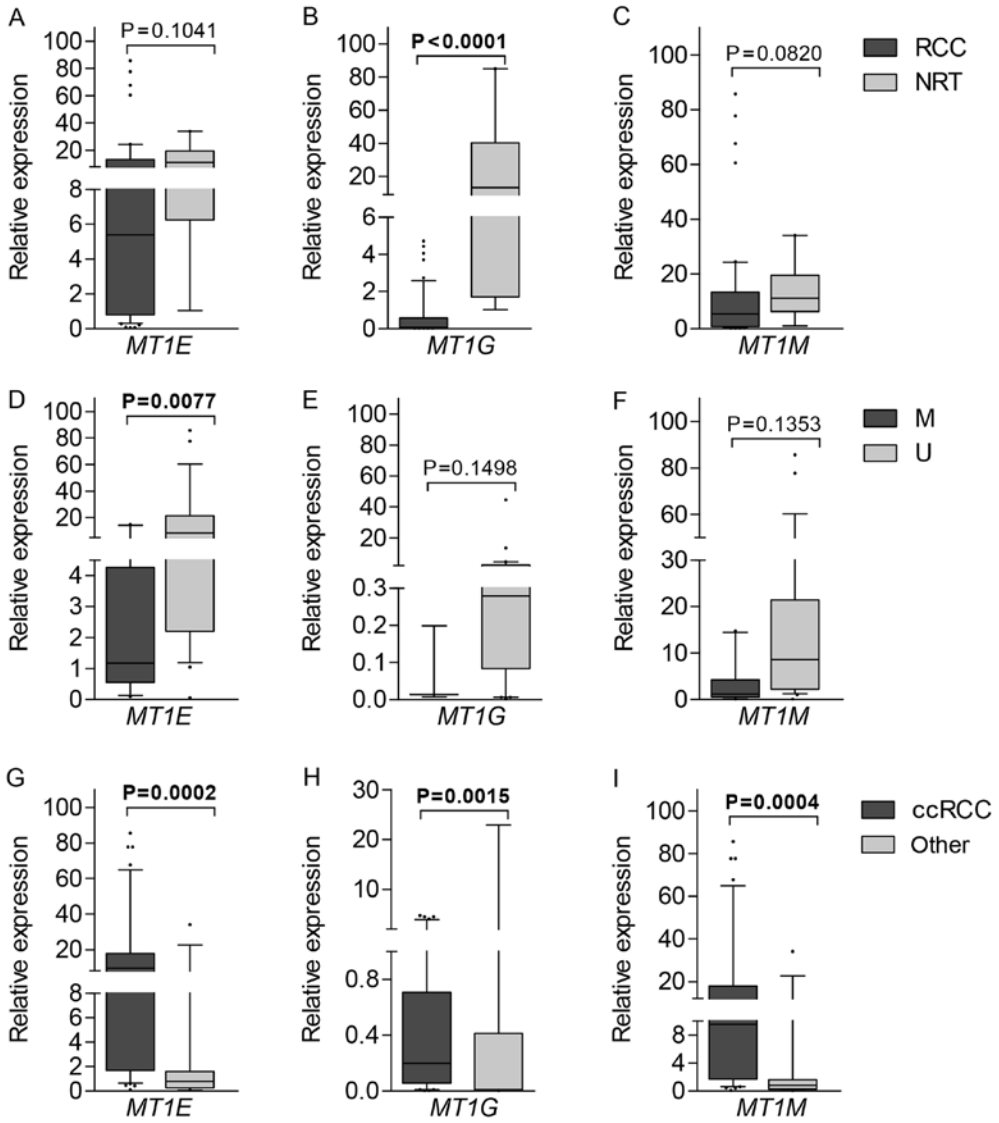

Figure 6. Relative expression of metallothionein genes in renal tissues. (A-C) Gene expression in renal tumors (RCC) and non-cancerous renal tissues (NRT). (D-F) Gene expression in renal tissues according to promoter methylation status. (G-I) Gene expression in RCC according to histological tumor subtypes. The box extends from the 25 to 75th percentiles; the line in the middle of the box is plotted at median; the whiskers represent the 10-90% range; data values outside the range are marked as dots. M/U, methylated/unmethylated gene promoter; ccRCC, clear cell RCC. Significant P-values are in bold.

was further analyzed according to clinicopathological patient characteristics. Methylation was more commonly detected in cases with advanced disease parameters. Higher *MT1E* methylation frequency was observed in tumors with higher differentiation grade (P=0.0144), while *MT1M* was more commonly methylated in RCC cases characterized with higher Fuhrman grade (P=0.0272; Fig. 5A and B). Tumors with methylated *MT1E* and *MT1M* promoters were significantly larger than those with unmethylated promoter status (P=0.0110 and P=0.0156, respectively; Fig. 5C). Furthermore, methylation of metallothionein genes was recurrently observed in tumors having necrotic zones; however, only *MT1E* showed significant difference (P=0.0449; Fig. 5D). No associations were detected between metallothionein gene methylation and patient age, sex, or pathological tumor stage (data not shown).

Gene expression analysis and association with DNA methylation. Gene expression at the transcriptional level was quantified by means of RT-qPCR. Lower expression levels of *MT1E*, *MT1G* and *MT1M* were detected in tumors as compared to NRT samples, however, only *MT1G* showed significant difference (P<0.0001; Fig. 6A-C). Downregulation of *MT1E* correlated with methylated promoter status (P=0.0077), while no such associations were detected for *MT1G* and *MT1M* (both P>0.0500; Fig. 6D-F). All three genes were expressed at significantly higher levels in ccRCC in comparison to the mixed group of other histological tumor subtypes (P=0.0002, P=0.0015 and P=0.0004 for *MT1E*, *MT1G* and *MT1M*, respectively; Fig. 6G-I). No other associations were observed between gene expression and clinical-pathological parameters.

Associations with metabolic syndrome-related parameters. Metabolic syndrome is concomitant with particular tumor types and is considered to be a risk factor of kidney cancer. In the present study, molecular alterations of metallothionein genes were associated with particular parameters used to diagnose metabolic syndrome. Methylated *MT1E* status in ccRCC subtype was more common in cases with raised fasting

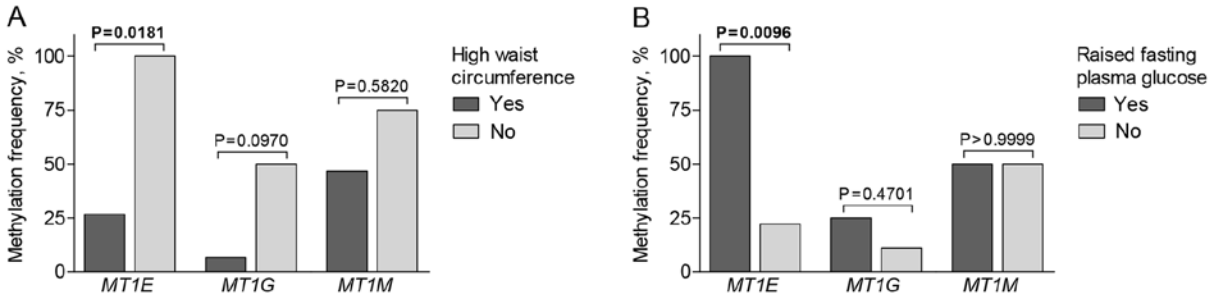

Figure 7. DNA methylation of metallothionein genes in renal clear cell tumors (ccRCC) according to metabolic syndrome-associated characteristics. (A) Methylation frequencies according to waist circumference. (B) Methylation frequencies according to fasting plasma glucose level. Significant P-values are in bold.

plasma glucose level (P=0.0096), but less frequent in patients with high waist circumference (P=0.0181; Fig. 7). Notably, expression of *MT1G* and *MT1M* was positively correlated with women's waist circumference (R_S=0.38, P=0.0490 and R_S=0.47, P=0.0119, respectively), whereas *MT1M* was upregulated in men having raised fasting glucose level (P=0.0348; data not shown). Due to the missing data for the majority of the cases, associations with other metabolic syndrome-related parameters (such as hypertriglyceridemia or high-density lipoprotein level) were not analyzed.

Discussion

In recent years, metallothioneins have emerged as important players in human carcinogenesis. Due to their unique function of metal ion buffering and delivery in a cell, these small cysteine-rich proteins have been shown to be pivotal regulators of various cellular processes, such as proliferation, differentiation, or apoptosis. Besides metal ion homeostasis and detoxification, metallothioneins protect cells against oxidative stress and DNA damage by scavenging free radicals. Numerous studies have reported deregulation of metallothionein expression in human tumors and, thus, their important role in various aspects of carcinogenesis has been proposed (10-15). However, the mechanisms responsible for the deregulated expression have been sparsely investigated.

In the present study, we investigated several protein-coding metallothionein genes in renal tumors aiming to evaluate their promoter DNA methylation for potential clinical utility. Four metallothionein genes, namely *MT1E*, *MT1G*, *MT1F* and *MT1M*, were selected for epigenetic analysis in renal cell carcinoma (RCC) and paired non-cancerous renal tissues. To the best of our knowledge, this is the first study to report aberrant methylation of *MT1E* and *MT1M* genes in RCC. We showed that methylation of *MT1E* and *MT1M* genes was tumor-specific, which indicates the potential clinical value of the two biomarkers in RCC diagnostics. Moreover, these results were supported by our preliminary observations made by analyzing the TCGA KIRC cohort (19). Until now, the two genes have been investigated in several other cancer types mostly at transcriptional and/or translational levels; however, the data concerning their promoter methylation are limited. In the present study, *MT1E* methylation associated with downregulated gene expression was observed in RCC, which is in accordance with our previous results obtained in prostate tumors (12). Other studies have also reported epigenetic silencing of *MT1E* in endometrial tumors, melanoma, and several other cancer localizations (22,23). The presence of *MT1E* methylation in metastases of melanoma patients, as well as in several invasive melanoma cell lines, suggest its potential involvement in cancer progression (23). In this study, *MT1E* was more frequently methylated in tumors of higher differentiation grade, larger size, or having necrotic zones, all of which are indicative of advanced disease. Furthermore, *MT1E* methylation status was associated with patient waist circumference and raised fasting plasma glucose, i.e. two of the parameters used for diagnosing metabolic syndrome, which is considered as one of the RCC risk factors. This hints that *MT1E* methylation may be an early event in renal carcinogenesis and, thus, lays the grounds for future investigations.

MT1M was the most commonly methylated gene in our cohort (43%). Its aberrant methylation was also observed in pericancerous renal tissues (PRT) (40%) indicating the field cancerization phenomena in RCC, i.e. when histologically normal tissue adjacent to cancer is primed to undergo transformation (24). Promoter methylation of *MT1E* and *MT1G* was also present in tumor-surrounding tissues suggesting that such epigenetic alterations might precede the development of RCC and predispose to multifocal tumors. In clinical practice, detection of aberrant methylation in normal-appearing biopsy specimens may be indicative of a missed cancerous lesion nearby and could justify the need for a repeat biopsy. Furthermore, together with *MT1E*, interfocal heterogeneity of *MT1M* methylation status was observed in several RCC cases, which is most likely attributable to discrepant grades of different tumor foci. To date, epigenetic analysis of *MT1M* in RCC has not been reported; however, it is one of the most studied metallothionein genes in various other cancer types. Downregulation of *MT1M* has been observed in hepatocellular, esophagus squamous cell carcinomas, breast, and other tumors and was associated with various clinicopathological parameters describing cancer aggressiveness (25-27). In this study, *MT1M* methylation was more frequently detected in tumors of larger size and higher Fuhrman grade, which is in accordance with previous observations in other tumors reporting its putative role in cancer progression (25,27,28).

In the present study, methylation of *MT1G* and *MT1F* was rare or absent. However, *MT1G* was the only metallothionein with significantly decreased gene expression in RCC as compared to healthy tissues. Aberrant *MT1G* methylation as a

novel biomarker of RCC was first reported by Dalgin *et al* (29). In previous studies, *MT1G* downregulation and epigenetic silencing have been associated with poor clinical outcome and/or drug resistance in various tumors (6,30,31). In addition, another mechanism, loss of heterozygosity, has been reported as being potentially responsible for the downregulated expression (32); however, it was not evaluated in the present study. We did not detect any correlations between *MT1G* promoter methylation or transcriptional expression and clinicopathological variables, which may be related to the low *MT1G* methylation frequency observed in our cohort. As no aberrant methylation of *MT1F* was detected in any renal tissues, this gene was omitted from our gene expression analysis. According to recent studies (5), *MT1F* overexpression rather than downregulation seems to be more commonly observed in cancer, which is in agreement with the lack of promoter methylation in our data.

Previous studies have demonstrated that metallothionein expression is quite specific to tumor localization as summarized by Si *et al* (5). Even in the same type of cancer, results can be contradictory due to the unique tumor microenvironment and varying external stimuli. In this study, despite promoter methylation status, *MT1E*, *MT1G*, and *MT1M* were expressed at significantly higher levels in ccRCC as compared to other histological subtypes of renal tumors. Considering their previously reported role in drug resistance, expression and/or methylation analysis of the three metallothionein genes in renal tumors could potentially provide additional information for treatment decision making.

In conclusion, this study has revealed the potential clinical value of aberrant promoter methylation of metallothionein genes in RCC. Methylated promoter status of *MT1E* and *MT1M*, together with other clinicopathological disease parameters, may serve for more accurate RCC characterization and personalized treatment selection at the time of diagnosis. However, further validation of these putative biomarkers are needed in larger independent cohorts, including liquid-biopsy samples, such as plasma or urine. DNA methylation analysis in body fluids not only enables patient monitoring by acquiring serial samples but is also considered to better reflect all tumor foci (including metastases), unlike tissue biopsy, which poorly accounts for RCC heterogeneity. As the diversity of metallothionein functions in the cell has been coming to light of late, functional analysis may provide significant insights on how the observed epigenetic deregulation is translated to the protein level, and also may indicate potential targets for drug development.

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Availability of data and materials

The RCC datasets analyzed in the current study are available from the corresponding author on reasonable request. The KIRC dataset used for general epigenetic overview of the metallothionein gene family is publicly available from the TCGA Research Network (http://cancergenome.nih.gov/).

Authors' contributions

RM performed the experiments and the data analysis, and drafted the manuscript. AZ and AB collected and analyzed the clinical data. FJ coordinated the patient selection, supervised the clinical data analysis and was involved in the conception of the study. SJ contributed to study implementation, supplied the samples, and participated in establishing research schemes. KD conceived and designed the study, supervised the experimental part of the study, and was a major contributor in writing the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Approval to conduct biomedical research (no. 158200-13-620-192) was obtained from the Lithuanian Bioethics Committee (Vilnius, Lithuania) before initiating the study, and all patients provided informed consent for participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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