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BRIEF REPORT

Expression and prognostic significance of MT1 isoforms in clear cell renal cell carcinoma [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Metallothioneins (MTs), a family of low-weight cysteine-rich proteins, play key roles in tumor biology, such as proliferation, differentiation, apoptosis, and drug resistance. Clinical studies have demonstrated that deregulation of MTs in various types of solid cancers. However, a comprehensive overview of MT1 isoforms expression and clinical relevance in clear cell renal cell cancer (ccRCC) is lacking. The present study explored mRNA expression levels and prognostic values of MT1 isoforms in ccRCC tissues using The Cancer Genome Atlas (TCGA), Gene Expression Profiling and Interactive Analysis (GEPIA) and Oncomine database. The study observed that mRNA expression levels of six members of MT1 isoforms decreased in renal cancer tumor tissues compared with normal tissues. We further found that high-expression of MT1G, MT1H, MT1F and MT1X was related with poor overall survival time in ccRCC patients and high-expression of MT1G, MT1F and MT1X were inversely associated with disease-free survival time in ccRCC patients. Based on the correlation analysis, MT1G was identified to be co-expressed with MT1H and MT1F in ccRCC tissues. These findings suggested that MT1 isoforms mRNA may serve as diagnostic and prognostic markers for ccRCC.

Keywords

Metallothionein, ccRCC, TCGA, Oncomine, Bioinformatics

This article is included in the **Oncology** gateway.

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2. Leili Saeednejad Zanjani ,

Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common type of renal tumor, which approximately constitutes 3% of adult malignancies and accounts for 90% of all renal malignancies^{1,2}. In recent years, the understanding of the genetics and biology of RCC had been improved dramatically, however, the mortality for patients with advanced or metastatic ccRCC is still high³⁻⁵. Therefore, it is essential to find new biomarkers to improve the diagnosis and prognosis of ccRCC patients.

Metallothioneins (MTs) are a family of cysteine-rich proteins. Their molecular weight ranges from 6 to 7 kDa and they play essential roles in tumor biology such as proliferation, differentiation, apoptosis, and drug-resistance⁶⁻¹¹. MT proteins are encoded by MT1-4 genes family located on chromosome 16q13¹², among them, MT2-MT4 are encoded by a single gene, while MT1 isoforms were encoded by a set of 14 genes, which consists of eight protein coding genes (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1M and MT-1X) and six pseudogenes (MT-1C, MT-1D, MT-1I, MT-1J, MT-1K and MT-1L)¹³⁻¹⁵. The expression of MT1 isoforms vary at cellular levels and in different tissues¹³. Changes in MT1 isoform expression were detected in various types of human malignancies¹⁴⁻¹⁸. Therefore, MT1 isoform profiling had been utilized as diagnostic markers and as prognostic predictors of tumor diseases¹²⁻¹⁴. Lots of work has been performed regarding the expression and prognostic value of MT1 isoforms in ccRCC¹⁹⁻²². However, there was still not enough information about the expression and prognostic value of the MT1 isoforms in ccRCC. Thus, it would be of great importance to elucidate the pattern of MT1 isoform expression and evaluate their prognostic value in ccRCC for patients' benefits.

The present study aimed to explore mRNA expression status of MT1 isoforms and their prognostic significance in ccRCC. For this purpose, we firstly investigated mRNA expression levels of MT1 isoforms in human ccRCC using The Cancer Genome Atlas (TCGA), Gene Expression Profiling and Interactive Analysis (GEPIA) and Oncomine database. Secondly, prognostic value of MT1 isoforms mRNA expression in ccRCC patients was evaluated through GEPIA by analyzing clinical data from the TCGA-KIRC project. Finally, correlations of MT1G with other MT1 isoforms were conducted using the R program to show the correlation in ccRCC.

Methods

Expression and survival analysis in GEPIA database

TCGA²³ is a publicly available and open access platform. As a result, ethics committee approval was not required for use of the data from this platform. GEPIA (Peking University, Peking, China) was used to assess the association between gene expression and prognosis^{24,25}. GEPIA adopts a standard processing pipeline to analyze the RNA-Seq expression data from GTEx and TCGA, which include 8,587 normal and 9,736 tumor samples. Here, we selected the MT1 family genes, including, MT1G, MT1F, MT1X, MT1H, MT1A, MT1E, MT1M, MT1L, and the cancer types (Kidney Renal Clear Cell Carcinoma; KIRC). For the gene mRNA level expression between normal and tumor tissue in ccRCC, a boxplot using disease state as a variable was graphed to calculate differential expression of MT1 isoforms. We obtained samples from TCGA and used GEPIA to analyze the connections

between overall survival and disease-free survival rates with MT1 isoforms expression in KIRC. Log-rank test was used to test the difference between Kaplan-Meier survival curves. Kaplan-Meier survival analysis was performed to analyze the relationship between the expression of MT1 isoforms and the survival days of KIRC patients²⁵. MT1s were entered into the database to obtain Kaplan-Meier survival plots. The log rank p values and hazard ratios were calculated. A p-value < 0.05 was considered statistically significant.

Oncomine database analysis

Analysis of MT1 isoform expression in ccRCC was performed using the Oncomine database (www.oncomine.org; Compedia Biosciences, Ann Arbor, MI, USA). Oncomine can be accessed through the Oncomine Research Edition using an institution supplied email address²⁶. We compared expression of the MT1 isoforms in clinical specimens of tumor datasets that contain both normal and tumor tissue. We further analyzed the MT1G gene expression in the six projects which contain both normal and tumor tissues of ccRCC. The six projects were Higgins Renal, Cutcliffe Renal, Gumz Renal, Jones Renal, Beroukheim Renal, Yusenko Renal and Lenburg Renal. Data were extracted to evaluate the expression of MT1 isoforms in RCC. In the study, p = 0.05, a fold change of 2, and a gene rank in the top 10% were set as the significance thresholds. Student's t test was used to analyze the difference in the expression of MT1s in RCC.

Dataset gene correlation analysis

Gene expression in KIRC from the TCGA dataset was obtained through the 'cgdsr' R package (version 3.6.2). The mRNA sequence data and corresponding clinical traits of the TCGA-KIRC project were downloaded from TCGA, which contained 537 ccRCC tumor tissues. Gene symbol annotation information was used to match probes with corresponding genes. The correlation of MT1G with other MT1 isoforms was calculated by cor function in R using Spearman correlation and visualized using ggplot2 (version 3.3.0) and ggpvr (version 0.2.5) packages in R (version 3.6.2) in R-Studio (version 1.2.5033). The code is supplied as *Underlying data*.

Results

mRNA expression of eight MT1 isoforms in ccRCC tumor and normal tissues

To evaluate the MT1 isoforms mRNA expression levels in ccRCC normal and tumor tissues, we firstly analyzed transcriptome data from the TCGA-KIRC project using GEPIA. We found that mRNA expression levels of six MT1 isoforms, including MT1G, MT1H, MT1F, MT1X, MT1E and MT1M were simultaneously decreased in ccRCC tumor tissues compared with normal tissues (Figure 1). The results indicated that six out of eight transcripts of MT1 isoforms were significantly downregulated in ccRCC.

To further confirm the expression levels of MT1 isoform mRNA in clinical samples, we screened the Oncomine database to investigate mRNA expression of MT1 isoforms between tumor and normal tissues. As shown in Figure 2A, eight MT1 isoform mRNA levels decreased in tumor tissues compared to normal tissues. In renal tumor cases, decreased mRNA expression of MT1 isoforms were detected in a total of nine datasets for MT1G,

Figure 1. Validation of MT1 isoform mRNA expression levels between normal and tumor tissue in clear cell renal cell carcinoma based on TCGA-KIRC data analyzed with GEPIA. (A) MT1G, (B) MT1H, (C) MT1F, (D) MT1X, (E) MT1E, (F) MT1M, (G) MT1L and (H) MT1A. The criteria thresholds for datasets were $\log_2FC > 1$; $*p < 0.01$.

seven for MT1F, five for MT1H and MT1X, four for MT1H, four for MT1E, and two for MT1M. Taken together, these results indicate that mRNA expression levels of MT1 isoforms are downregulated in renal tumor.

To investigate the decreased expression of MT1G mRNA expression level in ccRCC tissues, we investigated the differences in mRNA level of MT1G between tumor and normal tissues in ccRCC using the Oncomine database. As shown in Figure 2B, there were six ccRCC projects containing tumor and normal tissues and all the datasets indicated that the expression levels of MT1G dramatically decreased in tumor tissues. Taken together, these results from clinical specimens demonstrate that mRNA expression levels of MT1G are significantly downregulated in ccRCC tumor tissues.

Correlation between MT1G and MT1 isoforms in ccRCC

To figure out the MT1 isoform transcripts that are co-expressed with the MT1G transcript, we performed a correlation analysis between mRNA expression levels of MT1G and other MT1 isoform transcripts based on TCGA-KIRC transcriptome data (Spearman correlation shown in Figure 3). A strong correlation

of mRNA expression in cancer tissues ($r \geq 0.8$) was found between MT1G and MT1H, MT1G and MT1F, a moderate correlation ($r \geq 0.5$) existed between MT1G and MT1M, MT1X or MT1E. The expression of eight MT1 isoform mRNA and clinicopathological information involved in this study are supplied in *Underlying data*.

Prognostic relevance of eight MT1 isoforms transcripts in ccRCC

To assess the value of eight MT1 isoforms in ccRCC prognosis, the relevance between MT1 isoforms and overall survival time and disease-free survival time were investigated. First, we examined the prognostic relevance of eight MT1 isoform mRNA expression in ccRCC using GEPIA survival analysis based on TCGA-KIRC clinicopathological data. The patients from the TCGA-KIRC dataset ($n = 516$, which have both the transcriptome data and clinicopathological data) were classified into low- and high-expression groups according to the median of the specific gene mRNA expression level. For MT1G, the clinicopathological information of different MT1G expression groups is shown in Table 1; survival differences between the low and high expression groups were detected with Kaplan-Meier

Figure 2. Validation of MT1 isoform mRNA expression levels based on Oncomine database. (A) Expressions of MT1 isoform mRNA in 20 common cancers were compared with those in corresponding normal tissues. The search criteria thresholds for datasets of cancer versus normal analysis were p-value of <0.0001, a fold change of >2, and a gene rank in the top 10%. Red signifies gene upregulated in the analyses; blue represents gene downregulated. **(B)** Confirmation of the MT1G decreased expression in clear cell renal cell carcinoma tumor tissues compared to normal tissues based on the Oncomine database in different projects.

Figure 4. Overall survival analysis of eight MT1 isoforms in clear cell renal cell carcinoma (based on TCGA data in GEPIA). (A) MT1G, (B) MT1F, (C) MT1X, (D) MT1H, (E) MT1M, (F) MT1A, (G) MT1E and (H) MT1L. Red line represents samples with genes highly expressed and blue line for lowly expressed. HR: hazard ratio.

Figure 5. Disease free survival analysis of eight MT1 isoforms in clear cell renal cell carcinoma (based on TCGA data in GEPIA). (A) MT1G, (B) MT1F, (C) MT1X, (D) MT1H, (E) MT1M, (F) MT1A, (G) MT1E and (H) MT1L. Red line represents samples with genes highly expressed and blue line for lowly expressed. HR: hazard ratio.

Figure 6. Correlation between the expression levels of MT1 isoforms and the pathologic stage of clear cell renal cell carcinoma (based on TCGA data in GEPIA). (A) MT1G, (B) MT1H, (C) MT1F, (D) MT1X, (E) MT1E, (F) MT1M, (G) MT1A and (H) MT1L.

stage. In contrast, MT1F and MT1X showed positive associations between mRNA level and tumor stage, which were consistent with a previous studies that showed that MT1 isoforms are correlated with tumor stage^{12,18–20}. The data suggested that MT1 isoforms may possibly be used as a clinical prognostic parameter.

The MT1 gene family contains MT-1A, -1B, -1E, -1F, -1G, -1H, -1M and -1X and the rest of MT-1 genes (MT-1C, -1D, -1I, -1J and -1L) are pseudogenes^{12,13}. The expression and localization of individual MT1 isoforms and pseudogenes vary at cellular levels in individual tissues¹³. Based on the TCGA-KIRC transcriptome data, we couldn't detect mRNA expression of MT1D, MT1C, MT-1I and MT1J in ccRCC, and we detected low expression of MT1B in renal cancer tissues, which meant a tissue specific expression of the MT1 isoforms. Furthermore, we confirmed that MT1G expression is strongly correlated to MT1H and MT1F and moderately correlated to MT1M, MT1X and MT1E. Considering the expression profiles of MT1 isoforms vary in different cancer tissues, further exploration of MT1 isoform expression profiles and function in ccRCC should be addressed.

In conclusion, this, to the best of our knowledge, is the first systematic study on MT1 isoform expression profiling in ccRCC and evaluation of the relationship between MT1 isoforms and clinical pathological features. However, further studies are required to understand the mechanism of downregulation of MT1 isoforms and the roles MT1 isoforms play in ccRCC, which would be of great importance to ccRCC patients.

Data availability

Underlying data

Open Science Framework: Expression and Prognostics Significance of MT1 isoforms in Clear Cell Renal Cell Carcinoma, <https://doi.org/10.17605/OSF.IO/9XUND>³⁰.

This project contains the following underlying data:

- Supplementary File 1: The clinicopathological information of the TCGA-KIRC project.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Leili Saeednejad Zanjani

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In this study, Niu *et al.* represent a critical study aimed at investigating the Expression and prognostic significance of Metallothionein (MT1) isoforms in important subtypes of renal cell carcinoma (clear cell renal cell carcinoma (ccRCC)) using bioinformatics tools such as using The Cancer Genome Atlas (TCGA), Gene Expression Profiling and Interactive Analysis (GEPIA) and Oncomine database. They found that decreased expression of mRNA levels of six members of MT1 isoforms in renal cancer tumors compared with normal tissues. They also found that increased expression of MT1G, MT1H, MT1F, and MT1X with poor overall survival (OS) in patients with ccRCC and high expression of MT1G, MT1F, and MT1X were inversely associated with disease-free survival (DSS) in ccRCC patients. The authors concluded that MT1 isoforms mRNA may serve as diagnostic and prognostic markers for ccRCC.

In my opinion, the experiment design and the presentation of the data in the bioinformatics sections are good. The paper is generally well-written and structured. However, the following points should be addressed by the authors:

1. The authors need to define 'the GTEx' in the introduction section for the first time - "The Genotype-Tissue Expression".
2. The authors should define the abbreviations, especially in the bioinformatics terms in figure legends.
3. All figure legends are highly incomplete and needed to be improved. Each figure legend should have a title and then describe the results of each section briefly and separately (A to...). The authors need to describe the results briefly in all figure legends.
4. It is suggested the authors describe the limitation of this study at the end of the manuscript. For example not using the experimental study to confirm the results.
5. Figure 2, Part B must be presented separately from Part A. Because the graphs are not clear

and should be shown in a larger size.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cancer stem cells, Cancer Biomarkers.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 13 July 2022

<https://doi.org/10.5256/f1000research.25386.r142191>

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Peng Guo

Previously it was reported that DNA methylation of metallothionein (MT) genes are associated with the clinical features of renal cell carcinoma (RCC). In the present study, the authors analyzed the association between mRNA expression levels and prognostic values of MT1 isoforms in ccRCC tissues using The Cancer Genome Atlas (TCGA); Gene Expression Profiling and Interactive Analysis (GEPIA); and Oncomine database. They found that expression of MT1 isoforms decreased in ccRCC tissues, and high expression MT1 isoforms were associated with poor prognosis of ccRCC patients. These findings indicated that expression level of MT1 isoforms mRNA may serve as diagnostic and prognostic markers for ccRCC.

Minor points:

1. Please give a brief introduction of the function of metallothionein (MT) genes in cancer.
2. The expression of MT1 isoforms decreased in ccRCC tissues, but high expression MT1 isoforms were associated with poor prognosis of ccRCC patients, and expression of MT1 isoforms increased in higher tumor stages. Does this mean that MT1 isoforms may promote ccRCC progression while their expression decreases in ccRCC? Please explain and discuss. Thus, is it possible that the protein level of MT1 isoforms increase in ccRCC? Can you confirm this?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cancer biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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