Aberrant expression of metallothioneins in clear cell renal cell carcinomas

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Aim. To find candidate tumor suppressor genes among metallothioneins for clear cell renal cell carcinoma. Methods. Analysis of the microarray data, quantitative PCR. Results. We found three genes encoding metallothioneins that showed reduced expression in different types of renal tumors, using protocol of the cross-platform meta-analysis of microarray data with normalization on several reference genes. Decreased expression of the MT1G, MT1F, and MT1H genes in clear cell renal cell carcinoma was confirmed by qPCR. Conclusions. The MT1G, MT1F and MT1H genes as well as may be considered as the candidate tumor suppressor genes for ccRCC.

Keywords: metallothionein, renal tumors, candidate tumor suppressor gene, microarrays.

Introduction

Nowadays cancer is one of the major causes of death. Tumorigenesis is a result of disturbance of genome function under the influence of various factors. One of the reasons of appearance origin of the malignant cells is inactivation of the tumor suppressor genes by methylation, mutations or deletions. Metallothioneins are the small proteins, consisting of 61–68 amino acid residues and characterized by a high content of cysteine and complete absence of aromatic amino acids [1]. The genes of a metallothionein family are located on the 16q13 locus and this family consists of 10 functional isoforms [2]. There are four major genes among them: MT1 (seven functional genes MT1A, -1B, -1E, -1F, -1G, -1H, -1X), MT2A, MT3, and MT4. [The] MT1 and MT2A genes are expressed in different cell types; MT3 is expressed only in the brain and sexual organs, and MT4 only in stratified squamous epithelium in skin and tongue. Earlier it has been shown that only the MT1 and MT2A genes are involved in the regulation of embryogenesis as well as in other processes, such as interaction with metals, oxidative stress, signaling of cytokine and glucocorticoid hormones [3]. The function and expression of the MT family genes in human tumors were investigated thoroughly, however, the published results are often controversial for different types of tumors; so, the real contribution of these important molecules to the malignant cell transformation has not been elucidated yet. Overexpression of the MT family genes was reported for some tumors, such as melanoma, breast and lung cancers. In contrast, expression of MT family genes is decreased upon carcinogenesis in other types of tumors, such as colon and hepatocellular carcinomas [3]. The regulation of expression of the MT family genes occurs through methylation of the promoter region. The inactivation by methylation for the MT1H gene was demonstrated for the malignant tumors of prostate and liver [4]; for
the \textit{MT1G} gene - in hepatoblastoma [5], thyroid tumors of different histological types [3], and in the patients with squamous cell cancer of the esophagus [6] as well. The methylation of promotor region of the \textit{MT1G} gene also was shown, especially for primary renal cell carcinoma; however, the expression has not been studied, [7]. It was demonstrated that the \textit{MT1F} expression was lower in the colon tumors due to the loss of heterozygosity [8, 9]. Notably, the level of \textit{MT1F} gene expression in primary renal cell carcinoma remained unchanged [10].

Using the protocol of the cross-platform meta-analysis of microarray data with normalization on several reference genes, we found three isoforms of the \textit{MT1} gene, the expression of which is reduced in various types of the renal tumors. Expression of the \textit{MT1G}, \textit{MT1F}, and \textit{MT1H} genes in clear cell renal cell carcinomas (ccRCC) was examined by qPCR. Our data suggest that the \textit{MT1G}, \textit{MT1F}, and \textit{MT1H} genes may act as oncosuppressors in ccRCC.

\textbf{Materials and Methods}

\textit{Analysis of microarray and search [for] MT genes with low expression}

We performed the online search for Series and DataSet files, using \url{http://www.ncbi.nlm.nih.gov/geo} and the following keywords: clear cell renal cell carcinoma, normal human kidney, chromophil renal cell carcinoma, chromophobe renal cell carcinoma, and oncocytoma. Series and DataSet files contain records of the data sets, which were studied by the common platform. The platform is a description of the microchip. Record describes the type of tissue, the conditions under which a single sample has been processed, manipulated incurred, and measurement results for that sample. The series [file] brings together a group of similar samples and provides description of the study. Files “Series” are provided by the authors of the study and GEO personnel transform them into a DataSet (GDSxxx). DataSet files contain information on expression levels obtained experimentally on the microchip. Each DataSet file contains the available data for a set of samples, and it is possible to compare them biologically and statistically within a single file. Files Series contain metadata translated into a format similar to the files DataSet, using package GEOquery for R (\url{http://bioconductor.org/packages/1.8/bioc/html/GEOquery.html}). Due to the fact that individual DataSet files were created by different authors, using different microarrays and equipment, it is impossible to compare the data from different files without normalization. To compare different files we have used two reference genes for normalization. For this, the expression value for each sample was divided by the geometric mean value of the \textit{PPIA} and \textit{TBP} expression in the same sample [11]. Upon the analysis, the average value for expression of each gene was calculated, using scripts written in Perl, for tumors and normal tissue.

\textit{Total RNA isolation}

20 pairs of tumor samples and normal adjacent tissues were obtained from the National Cancer Institute of the National Academy of Medical Sciences. All tissue samples were diagnosed as the same histological type – ccRCC, which was classified, according to the American Joint Committee on Cancer (TNM) and nuclear atypia by Furman (Table 1). Total RNA was isolated, using RNeasy mini Kit (Qiagen, Valencia, CA), according to the manufacturer instruction. The quality of the isolated RNA was assessed by electrophoresis, using 1 \% agarose gels.

\textit{cDNA Synthesis}

1 \mu g of the total RNA was treated with the DNA-se for 30 min at 37\textdegree C, and the reaction was inhibited with 25 mM EDTA (Thermo Scientific, USA). CDNA synthesis was performed, using the Oligo (dT) – primers (a final volume of reaction was 20 \mu l), and the First Strand cDNA Synthesis Kit (Thermo Scientific, USA), according to the manufacturer protocols.

\textit{Quantitative PCR}

The real time PCR (qPCR) reactions were carried [out], using a mixture that contained 1 \times reaction mix SYBR Green (Thermo Scientific, USA), including polymerase, dNTPs, 0.4 \mu M of each primer and
500 ng of cDNA. [The] Reaction conditions were as follows: 95°C – 10 min, 40 cycles 95°C – 15 s, 60°C – 20 s, 72°C – 30 s. A reaction volume was 25 µl. CFX96 BioRad machine was used. The quality of the PCR product was checked by the analysis of melting curves. Reactions for different samples were normalized, using the amplification reference gene TBP (encoding TATA box binding protein), which shows the constant expression in different samples. The relative fold change was calculated, using the ΔΔCt method. The efficiency of amplification was calculated by the R-package software, version 2.12.1 [12]. The primers were selected, using OligoAnalyzer 3.1 software. For [the] MT1G amplification the following primers were used: direct (5’-GCTCTCTGTGCCGCTGGTGT -3 ‘) and reverse (5’-GCAGCAGCTGCACTTCTCCGAT -3’), product size was 167 bp. For [the] MT1H amplification – direct (5’-CCAACTGCTCCTGCGGTG -3 ‘) and reverse (5’-TCTGACGCCCCCTTGTGCAAACA -3’), the size of the product – 132 bp.

Statistical analysis
A comparison between the groups was done by using the independent samples t test. A value of P<0.05 was considered as statistically significant.

Results and Discussion
7 files that contain the data of gene expression in tumors and normal kidney tissue were found in the GEO database. In total, there were 9 ccRCC of stage 1 by Furman, 11 ccRCC of stage 2, 14 ccRCC samples of stage 3 as well as 55 chromophyle and 14 chromophobe kidney tumors, 14 oncocytomas, and 23 samples of normal tissue. The genes were selected that showed a decrease in the expression by more than 5 fold (P <0.05), compared with the normal tissue. Then the search for the MT family genes was performed, looking for the genes with reduced expression in all six tumor types. Three such genes – MT1G, MT1H, and MT1F – were found. There are the literature data showing changes in their expression during the development of various cancers, that is why we assumed that these genes might be suppressors for kidney tumors. For each patient, the tumor and normal tissue samples were paired. The normal tissue control samples were represented by the adjacent to the tumor but not malignantly transformed kidney tissues. To study the gene expression pattern, 20 pairs of samples (tumor/norma) from the renal carcinomas of varying degrees of malignancy were used (Table 1).

### Table 1

| N | TNM       | Stage |
|---|-----------|-------|
| 1 | T1bNxM0  | I     |
| 2 | T1bNxM0  | I     |
| 3 | T1aNxM0  | I     |
| 4 | T2aNxM0  | II    |
| 5 | T1aNxM0  | I     |
| 6 | T1aNxM0  | I     |
| 7 | T4NxMo   | IV    |
| 8 | T1bNxMo  | I     |
| 9 | T3NxMo   | III   |
| 10| T1aNxMo  | I     |
| 11| T1aNxMo  | I     |
| 12| T2aNxMo  | II    |
| 13| T1aNxMo  | I     |
| 14| T1aNxMo  | I     |
| 15| T2aNxMo  | II    |
| 16| T1bNxM0  | I     |
| 17| T1aNxM0  | I     |
| 18| T1bNxM0  | I     |
| 19| T1aNxMo  | I     |
| 20| T2aNxM1  | II    |

MT1G, metallothionein 1G

The MT1G expression decreased significantly (P=0.0047) in ccRCC samples, compared with normal tissue (Fig. 1), that is in line with the results for other types of tumors [3, 10, 13–15]. The main mechanism of the regulation of MT1G gene expression is associated with promoter hyper-methylation. Hyper-
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Methylation of promoter region of the MT1G was observed in hepatoblastoma, hepatocellular carcinoma, thyroid tumors of different histological types, esophageal squamous cell cancer and prostate tumors [3, 5, 6, 13, 16]. The rate of hyper-methylation of MT1G promoter showed correlation with poor prognosis for patients with hepatoblastomas. So, hyper-methylation of the MT1G promoter might be useful as a prognostic factor for the course of disease; moreover, demethylation of this gene in hepatoblastoma might suggest the positive effect in the treatment [5]. The level of MT1G promoter methylation in prostate tumors correlates with tumor progression stage, but not with stage by Gleason. The MT1G promoter hypermethylation was observed more frequently in prostate tumors that spread beyond the prostate capsule. So, the MT1G promoter methylation is associated with the aggressive prostate cancer [16].

The qPCR results showed that the MT1G gene expression also reduced in ccRCC compared with normal tissue. Thus, the MT1G gene may be a potential tumor suppressor for ccRCC. Moreover, it might be also used as a prognostic tumor marker.

MTIF, metallothionein 1F

Expression of the MTIF gene was significantly lower (P = 0.00077) in ccRCC compared with normal tissue (Fig. 2). Only in tumor number 17, there was a slight increase in the expression.

Earlier a significant reduction of the MTIF gene expression has been demonstrated only for colon tumors and hepatocellular carcinomas [8, 17]. Overexpression of MTIF in the RKO colon cancer cell line resulted in promotion of apoptosis and also prevented cell migration, invasion and adhesion in vitro. A reduced expression of the MTIF gene in colon tumors was observed, due to loss of heterozygosity (p = 0.001), whereas the methylation of CpG islands in the promoter region of the MTIF gene was observed only in colon cancer cell lines RKO and LoVo. So, MTIF is a potential tumor suppressor of colon cancers, which inhibited mainly, due to loss of heterozygosity [8]. When the HepG2 cell line was transfected with [an] expression vector pCMV-MT1F, the cells proliferated slower, compared with the control line HepG2. It was also reported that the MTIF expression at the mRNA and protein levels is significantly higher.

Fig. 1. Relative mRNA level of MT1G gene in ccRCC compared to normal tissue, assessed by qPCR.
in the normal tissue, than in hepatocellular carcinomas. Hence, MT1F may play an important role in gene therapy of hepatocellular carcinoma [17]. Earlier it has been shown that the MT1F expression levels in primary renal cell carcinoma remain unchanged in comparison with the control group [10].

According to the obtained data (microarray analysis with GEO and qPCR), expression of the MT1F gene significantly reduced in ccRCC. As the MT1F gene is the potential suppressor for colon tumors and hepatocellular carcinomas, and therewith it showed reduced expression in tumors of the kidneys, we suppose that the MT1F gene is the putative tumor suppressor for ccRCC and might be used as the tumor progression marker.

**MT1H, metallothionein 1H**

Expression of the MT1H gene significantly (P = 0.004) reduced in ccRCC compared with normal tissue (Fig. 3).

The main mechanism of the gene expression regulation is associated with the MT1H promoter hypermethylation. So, inter-connection between the promoter methylation and the gene MT1H expression was shown in prostate and liver tumors. The low MT1H expression was also associated with poor clinical outcomes for patients with both, prostate and liver cancers. De-methylation of promoter in cell lines in vitro restores the MT1H gene expression, leading to a slower cell proliferation, suppression of colony formation, and reduced ability of cells to migrate [4]. It was shown that MT1H can interact with euchromatin histone methyltransferase 1 (EHMT1) enhancing its methyltransferase activity on histone 3. Inhibition of the interaction between EHMT1 and MT1H leads to inactivation of the tumor suppression by MT1H [4]. As has been shown earlier, the primary renal cell carcinoma biopsy specimens had a lower expression of the MT1H transcripts than the pooled normal human controls [10].

**Fig. 2.** Relative mRNA level of MT1F gene in ccRCC compared to normal tissue, assessed by qPCR.
Our qPCR data of the MT1H gene expression in ccRCC type in comparison with normal tissue show the same trend. Since the MT1H functions as the growth suppressor for prostate and liver tumors, and therewith shows a lower expression in the primary renal cell carcinoma, we suppose that this gene is the tumor growth suppressor for ccRCC. The MT1H gene might be used also as a tumor marker.

Conclusions

Using the protocol of the cross-platform meta-analysis of microarray data with normalization on several reference genes we have found three genes of the MT family that showed reduced expression in different types of renal tumors. The expression of the MT1G, MT1F, and MT1H genes in ccRCC was also studied by qPCR. Taking into consideration that the MT1G gene is a potential suppressor for different types of tumors, the MT1F gene is a potential suppressor for colon tumors and hepatocellular carcinoma, and the MT1H gene is a potential suppressor for prostate and liver cancers, we suppose that these genes are potential suppressors for ccRCC and can be used as the tumor markers.

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Текст на русском языке:

Зміна рівня експресії металлотіонеїнів при світло-клітинній карціномі нирок

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Мета. Пошук потенційних генів-супрессорів серед металлотіонеїнів при розвитку світло-клітинної карціноми нирок. Методи. Біоінформатичний аналіз баз даних мікрочипів, кількісна ПЛР. Результати. Використовуючи біоінформатичні методи порівняння різних баз даних мікрочипів із застосуванням кросплатформенної нормалізації по декількох референтних генах, нами було виявлено три гени з родини металлотіонеїнів, що показують знижену експресію у різних типах пухлин нирок. Зниження експресії генів MT1G, MTIF та MT1H в зразках світлоклітинної карціноми нирок було продемонстровано методом кількісної ПЛР. Висновки. Гени MT1G, MTIF та MT1H є потенційними супрессорами росту пухлин нирок.

Ключові слова: металлотіонеїни, пухлини нирок, потенційний ген-супрессор росту пухлин, мікрочипи

Изменение уровня экспрессии генов металллотионеинов при светло-клеточной карциноме почек

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Цель. Поиск потенциальных генов-супрессоров светло-клеточной карциномы почек среди металллотионеинов. Методы. Анализ результатов микрочипов, выделение потенциальной РНК, количественная ПЦР. Результаты. Используя метод сравнения результатов микрочипов с применением кросплатформенной нормализации по нескольким референтным генам, нами были найдены три гена семейства металллотионеинов, что показывает снижение экспрессии у различных типов опухолей почек. Значения экспрессии генов MT1G, MTIF и MT1H в зразках светлоклеточной карциномы нирок были продемонстрированы методом кількісної ПЛР. Выводы. Гены MT1G, MTIF и MT1H являются потенциальными супрессорами роста опухолей почек.

Ключевые слова: металллотионеины, опухоли почек, потенциальный ген-супрессор.