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PPM1M and *PRICKLE2* are potential tumor suppressor genes in human clear-cell renal cell carcinoma

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Aim. *To investigate the expression levels of PPM1M and PRICKLE2 in clear-cell renal cell carcinomas (ccRCC) and propose a mechanism leading to the expression changes in tumor.* ***Methods***. *Analysis of GEO data, quantitative PCR (Q-PCR), bisulfite sequencing, methylation-specific PCR, deletion search.* ***Results***. *We found that the PRICKLE2 expression was down-regulated in 83 % of samples. Decreased expression of PPM1M was shown in 33.3 % of ccRCC samples. The promoters of PPM1M and PRICKLE2 were not methylated, and no deletions were found in their sequences.* ***Conclusions***. *Our data suggest that PRICKLE2 and PPM1M might be candidates for the tumor suppressor genes in ccRCC.*

Keywords: renal cell carcinoma, genetic and epigenetic regulation, quantitative real time PCR, deletion, methylation status.

Introduction. Kidney cancer is a malignant tumor mainly of epithelial origin and is derived from the cells of proximal convoluted tubule. Clear-cell renal cell carcinoma (ccRCC) accounts 70–80 % of all renal cancer cases and is characterized by chemo- and radio-resistance [1]. According to the NotI-microarrays, the *PPM1M* and *PRICKLE2* genes showed the presence of methylation/deletion more than in 20 % of ccRCC tumor samples [2]. We performed an analysis of the GEO data base for the *PPM1M* and *PRICKLE2* genes (http://www.ncbi.nlm.nih.gov/geoprofiles/) and investigated their expression in ccRCC. An exact physiological role and function of *PPM1M* and *PRICKLE2* in human's carcinogenesis is not known for now. It is known, however, that encoded proteins are the members of cancer-related pathways. *PPM1M* inhibits the IL-1-NF-kappaB signaling pathway by selective dephosphorylation of IKKbeta [3]. *PRICKLE2* is involved in the WNT/planar cell polarity (PCP) signaling pathway that controls tissue polarity and cell movement [4]. These genes are tumor suppressor candidates in cervical cancer, non-small cell lung cancer and ovarian cancer [5–7]. It is possible, that these two genes might be potential tumor-suppressor genes in ccRCC as well. The present paper is devoted to a detailed study on the *PPM1M* and *PRICKLE2* gene expression in ccRCC.

Materials and methods. *Tissue samples*. Surgically removed tumors and surrounding rim of normal tissue were obtained from Kyiv National Urological Center (Ukraine). All tumor specimens were assessed according to the WHO International System of Clinico-Morphological Classification of Tumors (TNM). All samples were obtained according to the guidelines of the Ethical Committee of IMBG.

Isolation of DNA and total RNA. DNA samples were isolated, using GeneJET Genomic DNA Purification

Kit («Fermentas», Lithuania). Total RNA was isolated, using RNeasy Mini Kit («QIAGEN», USA). cDNA was synthesized, using RevertAid First Strand cDNA Synthesis Kits («Fermentas»). All procedures were performed according to the manufacturer's recommendations.

Analysis of gene expression levels. The analysis of relative expression of *PPM1M* and *PRICKLE2* genes was performed with the help of Q-PCR, using Master mix SYBR Green («Fermentas») and IQ5 Cycler («Bio Rad», USA) [8]. The primers were designed, using the Primer3 (http://frodo.wi.mit.edu/primer3/) and IDT (http://eu.idtdna.com/site) programs (Table 1). TBP was used as a reference gene [9].

Methylation status analysis. The analysis of methylation status of the *PPM1M* and *PRICKLE2* promoter regions was performed by methyl specific PCR as was described previously [10].

Deletion search for the *PPM1M* and *PRICKLE2* genes was performed, using PCR with primers for deletion search from NCBI database (Table 1) and subsequent analysis of products length in denaturing PAAG electrophoresis. PCR was performed under the standard conditions [10].

Statistical analysis was performed as described previously [8].

Results and discussions. In the current study 19 samples of ccRCC at the stage 1–4 and corresponding «normal» tissues were analyzed. The mean age of patients was 56 ± 3.75 (in the range of 46–69 years), with a male-to-female ratio of 58 % and 42 % respectively (Table 2). The samples were grouped according to the stage of tumor development and arranged in order of increasing atypia (Fig. 1, 2, Table 2).

The relative *PPM1M* gene expression analysis by Q-PCR allowed us to separate the tumor samples into three groups – with low, unchanged, and a high gene expression. The increased PPM1M expression was detected in 50 % of the samples (9 of 18). The expression of *PPM1M* was decreased in 33.3 % (6 out of 18) samples. In 16.7 % of cases (3 out of 18) statistically significant changes were not found.

*PRICKLE*2 was downregulated in the majority of samples (83 %, 15 out of 18). No correlation between age, sex, or stage of atypia was observed for both studied genes. (Fig. 2, Table 2).

In order to explain the gene expression changes, promoter methylation was investigated, using MSP PCR. No correlation between the gene expression level and *PPM1M* promoter methylation status was found. Moreover, there were no CpG islands in the promoter area of the *PRICKLE2* gene.

To study the putative deletions as one of the reasons of expression changes, we chose two pairs of primers (D3S1287, D3S4182) for *PRICKLE2* using NCBI database. No homozygous deletions were identified. Four pairs of primers for the *PPM1M* gene (A002C09, MARC_15661, RH76369, RH12810) were also selected (Table 1). No homozygous deletions were identified. Further, primers for sequence that contained CA repeats were selected independently. All samples were homozygous for the indicated loci; that means that a study on heterozygous deletions was not possible. No homozygous deletions were identified.

As a result of our work no deletions or promoter methylation of *PPM1M* and *PRICKLE2* were found. This corresponds to a low percentage of methylation/deletion changes in the microarray analysis of these genes [2].

The *PRICKLE2* gene encodes the prickle-like 2 protein that is one of the essential proteins in the Wnt-pathway, determining planar cell polarity (PCP). Thee prickle-like protein 2 participates also in the remodeling of cytoskeleton, change of cellular adhesion and mobility [11]. In this study we showed down-regulation of *PRICKLE2*. This may be associated with dedifferentiation processes and loss of the typical morphology of cells in the malignant transformation.

The *PPM1M* gene encodes a protein with phosphatase activity. A function of this protein was studied only in mice. However, it is known, that Ppm1m participates in the IL1-induction pathway by NF-κB. The Ppm1m dephosphorylates IκB that blocks activation of NF-κB, preventing the induction of inflammation-related proteins [3]. Therefore, the highly expressed *PPM1M* gene might be involved in the formation of active inflammatory lesion that surrounds neoplasm. Our data show that the *PPM1M* expression in ccRCC can be reduced as well as increased or remain unchanged, that might indicate a variety of ways to block NF-κB in tumors.

The absence of correlation between the expression decrease and methylation or deletions suggests that the

Table 1
Primers for target and reference genes to study expression levels, deletion search and methylation analysis

Primer's title (name)	Primer's sequence	Primer's title (name)	Primer's sequence
PPM1M (expression)	for 5'-GATGTACTGTCCAACGAGCAG-3' rev 5'-TGTCTTCCTTTCCCTGTGTG-3'	MARC_15661	for 5'-CTGGGAACACTGGCTGTCTC-3' rev 5'-GTAGGACACCTGCCCTTCCT-3'
PRICKLE2 (expression)	for 5'- TGCCCTATTGAGGAGAAGGA-3' rev 5'-TAATGGTTGTGATGGAGGAAT-3'	RH76369	for 5'-TTGAGATGGATGTGTGTTGAGG-3' rev 5'-GAAGGGCAGGTGTCCTACG-3'
TBP (expression)	for 5'-GAACCACGGCACTGATTTTC-3' rev 5'-CACAGCTCCCCACCATATTC-3'	RH12810	for 5'-GTTGGGGCATCAGACCAG-3'rev 5'-GGGTAGAGCACAAGGGACAA-3'
D3S1287	for 5'-ATAACACAACAAGCAAGCCTATGGT-3' rev 5'-GAGTGACATTTGCCCCTTTG-3'	PPM1M-CA	for 5'-CAGCACCTTATCCCCA-3' rev 5'-TGGGATGACTTGCTGTGT-3'
D3S4182	for 5'-AAGTGTTCAGAACAGTCTCTGGC-3' rev 5'-AACAAAACCTCAAAGGGCCT-3'	PPM1M-MSP Met	for 5'-CGT GTT TTA TCG ACG GTT TC-3'rev 5'-AAC GTA CGT CCT CGT ACG AA-3'
A002C09	for 5'-GTTAAGAGGCAGGCTACTAC-3' rev 5'-CTGAAAAGAGACCAGTTC-3'	PPM1M-MSP Unmet	for 5'-AGT TTTGTGTTTTATTGATGGTTTT-3' rev 5'-CCAAAATAACATACATCCTCATACA-3'

Table 2
Clinical characteristics and relative expression level (RE) of PPM1M and PRICKLE2 genes in ccRCC patients

Sample N	Sex	Stage of atypia	Age	TNM classification	PPM1M, RE	PRICKLE2, RE
1	F	1	49	T2N0M0	0,367 ± 0,062	No signal
2	M	1	57	T2N0M0	3,11 ± 0,437	0,549 ± 0,001
3	M	1	49	T2N0M1	2,353 ± 0,012	0,442 ± 0,017
4	M	1	57	T2N0M0	0,177 ± 0,014	2,667 ± 0,045
5	F	1	57	T2N0M0	4,116 ± 0,272	0,632 ± 0,033
6	F	1	73	T2N0M0	0,521 ± 0,003	1,613 ± 0,090
7	M	1	61	T2N0M0	0,991 ± 0,031	0,340 ± 0,035
8	M	1	50	T2N0M0	1,747 ± 0,009	0,514 ± 0,031
9	F	1	57	T2N0M0	1,062 ± 0,033	0,453 ± 0,080
10	F	2	69	T2N0M0	6,299 ± 0,078	0,102 ± 0,002
11	F	2	50	T2N0M0	0,279 ± 0,121	0,213 ± 0,007
12	M	2	55	T2N0M0	0,048 ± 0,010	0,118 ± 0,052
13	F	2	66	T2N0M0	0,919 ± 0,114	1,882 ± 0,484
14	M	2	49	T2N0M0	2,028 ± 0,020	0,141 ± 0,003
15	M	2	49	T2N0M0	No signal	0,630 ± 0,094
16	M	3	46	T3N0M0	1,86 ± 0,265	0,508 ± 0,020
17	M	4	51	T2N1M1	1,318 ± 0,147	0,618 ± 0,014
18	M	4	58	T3N0M0	4,173 ± 0,605	0,205 ± 0,014
19	F	4	61	T3N0M0	0,715 ± 0,073	0,025 ± 0,005

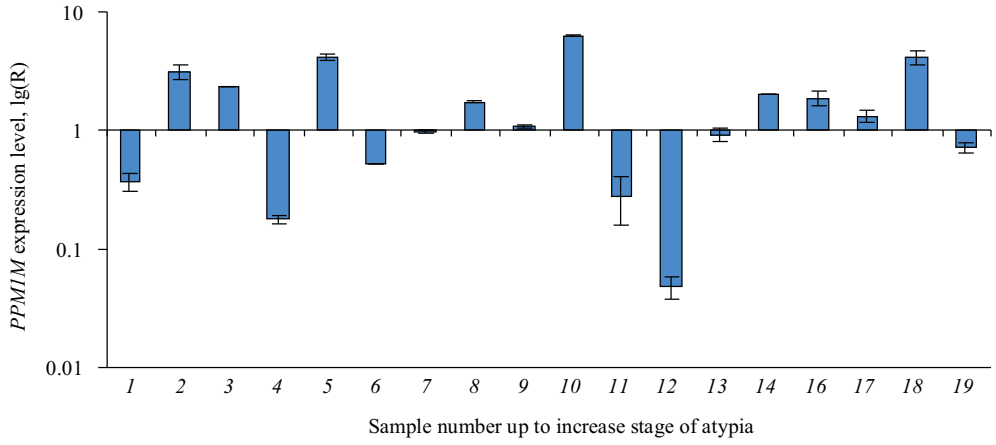

Fig. 1. Relative expression of *PPM1M* (*n* = 18) in ccRCC. Samples *1–14* – stage 1–2; *16–19* – stage 3–4

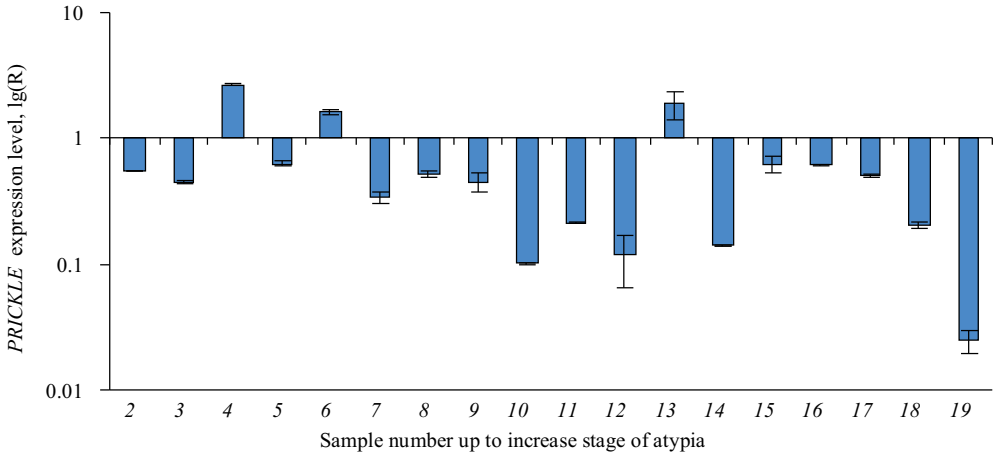

Fig. 2. Relative expression of *PRICKLE2* (*n* = 18) in ccRCC. Samples *2–14* – stage 1–2; *15–19* – stage 3–4

changes in *PPM1M* and *PRICKLE2* gene expression in ccRCC might be achieved by other mechanisms. It is possible that miRNAs or other proteins might be involved in regulation of mRNA expression.

Conclusions. For the first time the *PPM1M* and *PRICKLE2* genes expression has been analyzed in ccRCC by the Q-PCR method. In the present work, we have found, that the *PPM1M* expression is increased in 50 % of the samples. The expression of *PRICKLE2* was downregulated in 83 % of samples. Neither deletions nor methylation were a reason for a decline of the *PPM1M* and *PRICKLE2* expression. This indicates that another mechanism, that is not associated with DNA, aberrations is involved in the expression changes. Taking into consideration the functions of proteins, that are encoded by the *PPM1M* and *PRICKLE2* genes, we can suggest their involvement in the kidney carcinogenesis. Based on obtained results, the *PRICKLE2* gene might be a candidate for a tumor suppressor gene. Further studies are required to explain the mechanisms of detected changes in the *PPM1M* and *PRICKLE2* expression.

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PPM1M і *PRICKLE2* як потенційні гени – супресори пухлин у світлоклітинній карциномі нирки людини

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Резюме

Мета. *Дослідити рівень експресії PPM1M і PRICKLE2 у світлоклітинних карциномах нирки (ccRCC) і запропонувати механізм, що призводить до змін експресії генів у пухлинах.* ***Методи***. *Аналіз баз даних GEO, кількісна ПЛР (Q-ПЛР), бісульфітне секвенування, метил-специфічна ПЛР, пошук делецій.* ***Результати***. *Ми виявили, що експресія гена PPM1M знижена у 33,3 % зразків ccRCC, у той час як гена PRICKLE2 – у 83 % зразків ccRCC. Метилювання*

промоторної зони і делеції в генах PPM1M і PRICKLE2 не виявлено. ***Висновки****. Наші дані вказують на те, що PRICKLE2 і PPM1M можуть бути кандидатами в гени-супресори для ccRCC.*

Ключові слова: світлоклітинна карцинома нирки, генетична і епігенетична регуляція, кількісна ПЛР у реальному часі, делеції, статус метилювання.

PPM1M и *PRICKLE2* как потенциальные гены – супрессоры опухолей в светлоклеточной карциноме почки человека

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Резюме

Цель*. Исследовать уровень экспрессии PPM1M и PRICKLE2 в светлоклеточных карциномах почки (ccRCC) и предложить механизм, ведущий к изменениям экспрессии генов в опухолях.* ***Методы****. Анализ баз данных GEO, количественная ПЦР (Q-ПЦР), бисульфитное секвенирование, метил-специфическая ПЦР, поиск делеций.* ***Результаты****. Мы выявили, что экспрессия гена PPM1M снижена в 33,3 % образцов ccRCC, тогда как гена PRICKLE2 – в 83 % образцов ccRCC. Метилирование промоторной зоны и делеции в генах PPM1M и PRICKLE2 не обнаружены.* ***Выводы****. Наши данные показывают, что PRICKLE2 и PPM1M могут быть кандидатами в гены-супрессоры для ccRCC.*

Ключевые слова: светлоклеточная карцинома почек, генетическая и эпигенетическая регуляция, количественная ПЦР в реальном времени, делеции, статус метилирования.

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