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Heterozygous deletions are main cause of expression alterations of *PPM1M* and *PRICLE2* genes in human clear cell renal cell carcinomas

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Aim. To discover the mechanisms of the *PPM1M* and *PRICKLE2* genes expression alterations in clear cell renal cell carcinomas. **Methods.** Study of the copy number was performed, using the quantitative PCR (Q-PCR). **Results.** Deletions of *PPM1M* were found in 55 % of cases, amplifications – in 17 % and in 28 % of samples there were no changes of the copy number. We found the deletions of *PRICKLE2* in 50 % of samples, no changes of the copy number in 39 %, and amplifications in 11 % of cases. **Conclusions.** By the analysis of the gene copy number we have shown that homo- and heterozygous deletions are the main reason of changes in expression of the *PPM1M* and *PRICKLE2* genes.

Keywords: clear cell renal cell carcinoma, heterozygous deletions, quantitative PCR, copy number analysis.

Introduction

Annual incidence of tumors of the genitourinary system is over 200 thousand worldwide. During 2011 the kidney cancer was diagnosed in 5622 patients in Ukraine; 2469 of them died. For a quarter of these patients a period from the time of diagnosis to death is very short, less than one year (see http://www.ncru.inf.ua/publications/BULL_14/PDF_E/49-50-poch.pdf). Among urologic tumors, clear cell renal cell carcinoma (ccRCC later on in the text) takes the third place in incidence, following prostate and bladder cancer. ccRCC is the first by the number of death. ccRCC is the prevalent malignancy of the kidney, accounting for about 85 % of kidney cancers [1]. Kidney carcinoma is associated with the most negative prognosis and responds poorly to medical treatment, as a rule [2]. The course of disease and treatment depends on the molecular profile of tumors. Very important is to study the expression pattern of tumor suppressor genes (*TSG*) that play an important role in cancerogenesis. The *TSG* expression is usually downregulated in tumors by different mechanisms, such as genetic and epigenetic alterations. One of the most frequent aberrations in solid tumors is inactivation of *TSG* in chromosome 3 [3]. Using the NotI-microarray to study ccRCC samples, we have found that the *PPM1M* and *PRICLE2* genes showed genetic and/or epigenetic changes [4]. It has been already shown that these genes play the role of *TSG* in cervical, ovarian and lung cancers [5–7].

Earlier we have shown that a reduction in the *PPM1M* and *PRICKLE2* expression is typical for ccRCC; no promoter methylation was found for these genes, however [8]. It is known that these proteins are

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involved in the signalling pathways associated with carcinogenesis. In particular, PPM1M inhibits the IL-1-NF-kappaB signalling pathway by selective dephosphorylating of IKKbeta [9]. PRICKLE2 is involved in the WNT pathway of planar cell polarity determination (PCP), which regulated the polarity and movement of cells [10]. Therefore, there is a possibility that these two genes might be *TSG*s in ccRCC.

In the present work we performed a detailed study of heterozygous deletions of the *PPM1M* and *PRICKLE2* genes in ccRCC and propose the new mechanism of deletion of these genes during carcinogenesis.

Materials and Methods

Tissue samples. Samples of surgically removed tumors and surrounding tissues (considered as normal) were obtained from Kyiv National urology center (Kyiv, Ukraine). All tissue samples were characterized according to the International System of Clinical and morphological classification of tumors (TNM) [11] and WHO classification criteria [12]. The average age of patients was 48.59 (in the range of 30–68 years). Normal tissues were obtained at a distance of at least 2 cm from the tumor and histologically confirmed as unchanged renal epithelial cells. Altogether, 18 samples of ccRCC were investigated, i.e. 12 samples at stage 1–2 and 6 samples at stage 3–4.

Isolation of genomic DNA

Genomic DNA was isolated, using DNA purification Kit (Fermentas, Lithuania), according to the manufacturer's re-commendations. Quality of genomic DNA was as-

Table 1. **Summary of analysis on gene copy number in ccRCC samples**

Sample number	Sex	Stage of atypia	Age	TNM classification	Copy number of *PPM1M*	Copy number of *PRICKLE2*
1	M	1	38	T2NxM0	0.979 [0.979; 0.981]	*0.409 [0.408; 0.409]*
2	M	1	47	T2NxM0	**2.761 [2.720; 2.801]**	0.979 [0.979; 0.980]
3	F	1	43	T3N0M0	*0.677 [0.614;0.740]*	1.287 [1.149;1.426]
4	M	1	38	T2NxM0	1.212 [1.045; 1.377]	*0.848 [0.781; 0.914]*
5	F	2	46	T2NxM0	*0.759 [0.707;0.811]*	*0.809 [0.790;0.829]*
6	F	2	46	T2NxM0	0.933 [0.897;0.970]	1.009 [0.911;1.108]
7	M	2	41	T3NxM0	*0.706 [0.439; 0.973]*	*0.808 [0.729; 0.888]*
8	M	2	50	T2NxM0	0.973 [0.935; 1.011]	**1.631 [1.575; 1. 686]**
9	F	2	45	T2NxM0	**2.418 [1.947;2.889]**	*0,579 [0,528; 0,631]*
10	F	2	66	T3N0M0	*0.507 [0.406: 0.608]*	0.933 [0.879; 0.989]
11	M	2	68	T2NxM0	*0.631 [0.591; 0.671]*	*0.470 [0.465;0.474]*
12	M	2	38	T2NxM0	*0.366 [0.318; 0.415]*	*0.431 [0.363; 0.501]*
13	M	3	45	T2NxM0	*0.847 [0.814; 0.880]*	1.044 [0.850; 1.238]
14	M	3	67	T2NxM0	*0.398 [0.374; 0.375]*	1.025 [0.970; 1.080]
15	M	3	46	T2N0M0	*0.705 [0.701; 0.708]*	1.146 [0.989; 1.303]
16	F	4	61	T3N0M0	0.884 [0.690; 1.077]	**1.429 [1.261; 1.597]**
17	M	4	51	T3N0M0	**1.675 [1.405; 1.945]**	*0.525 [0.509; 0.541]*
18	M	4	62	T2NxM0	***0.223 [0.213; 0.233]***	***0.368 [0.314; 0.422]***

Note: Samples, which displayed ratio <0.35 and where gene was considered as homozygously deleted are marked ***bold italic***. Samples, which displayed ratio <0.85 and ≥0.35 and where genes were considered as heterozygously deleted are shown in *italic*. Samples, which had ratio >0.85 and where genes showed unchanged copy number are marked regular text Samples, which displayed ratio >1.5 and where genes were amplified marked **bold**.

sessed by agarose gel electrophoresis; DNA concentration was measured, using a spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies Inc., USA). The samples used for the Q-PCR reactions were of high molecular weight (OD 260/280 was in range 1.6–1.8).

Analysis of gene copy number. The Q-PCR method and Bio-Rad iQ5 machine to quantify the number of gene copies were used. It was shown that this method is accurate enough to determine the number of allele copies. Each Q- PCR reaction contained 12.5 ul 2xSYBR Green PCR Master Mix (Fermentas, Lithuania), forward and reverse primers at a concentration of 400 nM, genomic DNA – 10 ng/ml, and sterile water to make a final volume 25 ul. Gene *TBP* was used as the reference [13]. The primer design was carried out, using a program Primer3 (http://frodo.wi.mit.edu/primer3/) and Integrated DNA Technologies, http://eu.idtdna.com/ analyzer/Applications/Oli goAnalyzer/). The following primers were used (Invitrogen):

PPM1M_copy

LEFT PRIMER 5’-TCCGAATCCTGACAACTTC-3’
RIGHT PRIMER 5’- CCGAATGCTGGAACT CTA-3’

PRICKLE2_copy

LEFT PRIMER 5’-ATGTTTGCTCCTGGTTT CA-3’
RIGHT PRIMER 5’-ACTCAGACCCTCAGTTCAC-3’

TBP_copy

LEFT PRIMER 5’-ATAAAGCAGCAGCAGG GTTT-3’
RIGHT PRIMER 5’-AGTCAGTGTGGCAAACTTCG-3’

PCR was performed at the following conditions: denaturation at 95 °C for 10 min, after this 35 cycles: denaturation at 95 °C for 20 sec, annealing at 60 °C for 20 sec, and elongation at 72 °C for 40 sec.

Analysis of the number of copies of the *PPM1M* and *PRICKLE2* genes was performed by 2-ΔΔCP relative quantitative analysis [14].

According to the histological analysis, the contamination of tumour samples by normal stroma lymphocytes could be up to 30–40 %. Therefore, the homozygous deletions were considered when the calculated values were below 0.35; the heterozygous deletions were proposed if the calculated values were below 0.85. The locus was considered as amplified if the smallest of the range exceeded 1.5 [15].

Results and Discussion

An analysis of the gene copy number was performed for 18 samples of ccRCC and corresponding to them normal tissues (Table 1).

The *PPM1M* gene was deleted in 55.6 % of cases (nine cases of heterozygous deletions and one homozygous deletion). Amplification were observed in 16.7 % (three samples), while 27.8 % cases were unchanged (five samples). Previously we have reported that the expression level of *PPM1M* altered in 50 % of cases in ccRCC tumors [8].

The product of *PPM1M* gene is a serine threonine phosphatase from the PPM family (Mg^{2+}- and Mn^{2+}-dependent). Phosphorylation is an important post-translation modification that regulates many signaling systems and it is an effective way to control cellular internal and external signal responses [16]. Phosphatases together with kinases regulate phosphorylation and are involved in many physiological processes, such as cell migration, proliferation, intracellular substrate localization, apoptosis, differentiation, metabolism, and immune system response [17].

Phosphatases are also involved in the cell cycle regulation [18], therefore they are considered as suppressors of tumor growth. Most probably, they should be inactivated in the process of carcinogenesis. One of the functions of *PPM1M* is the inhibition of the IL-1-induced activation of NF-kappa-B. The IL-1 is a cytokine that has different functions in control of inflammation. [9].It is yet un known how *PPM1M* is involved in cancerogenesis [19].

Deletions in the *PRICKLE* gene were found in 50 % of samples (eight samples heterozygous deletions and one homozygous deletion). In 38.9 % (seven samples) there were no changes in the copy number, while amplification was observed in 11.1 % of cases (two samples). According to our previous data, the expression level of *PRICKLE2* altered in 83 % of cases [8].

The PRICKLE2 protein is a member of WNT-cascade that activates Fz-receptors on the cell surface. This pathway regulates the planar polarization of the cells that differ from the basal-apical polarization and is, actually, perpendicular to the latter [20]. Apart that the PRICKLE2 is attached to the inner surface

of the cell membrane by another protein – PCP- strabismus, not much is known about its function [21].

Conclusions

Previously, we have shown that the expression of *PRICKLE2* and *PPM1M* altered in ccRCC. In the present paper by the analysis of the gene copy number we have shown that homo- and heterozygous deletions are the main reason of changes in the expression of these genes.

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Гетерозиготні делеції – основна причина змін експресії генів *PPM1M* та *PRICKLE2* у світлоклітинних карциномах нирки людини

Мета. Знайти механізм, відповідальний за зміну експресії генів *PPM1M* і *PRICKLE2* у світлоклітинних карциномах нирки людини. **Методи.** Вивчення змін кількості копій генів було проведено за допомогою кількісної ПЛР (Q- ПЛР). **Результати.** Делеції гена *PPM1M* були знайдені у 55,6 % випадків, ампліфікації – у 16,7 %, а у 27,8 % зразків не було змін кількості копій гена. Ми виявили делеції гена *PRICKLE2* у 50 % зразків, зміни не виявлено у 38,9 % зразків, а ампліфікації спостерігалися у 11,1 % випадків. **Висновки.** За допомогою аналізу кількості копій гена ми показали, що гомо- та гетерозиготні делеції є головною причиною зміни експресії генів *PPM1M* і *PRICKLE2*.

Ключові слова: світлоклітинна карцинома нирки, гетерозиготні делеції, кількісна ПЛР, аналіз кількості копій гена.

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Гетерозиготные делеции являются основной причиной изменения экспресии генов *PPM1M* и *PRICKLE2* в светлоклеточных карциномах почки человека

Цель. Найти механизм, ответственный за изменение экспрессии генов *PPM1M* и *PRICKLE2* в светлоклеточных карциномах почки человека. **Методы.** Изучение изменений количества копий проводили методом количественной ПЦР (Q-ПЦР). **Результаты.** Делеции гена *PPM1M* были найдены в 55,6 % случаев, амплификации – в 16,7 %, а в 27,8 % образцов не было изменений количества копий гена. Мы обнаружили делеции гена *PRICKLE2* в 50 % образцов, в 38,9 % образцов изменений не обнаружено, амплификации наблюдались в 11,1 % случаев. **Выводы.** С помощью анализа количества копий гена мы показали, что гомо- и гетерозиготные делеции являются главной причиной изменения экспресии генов *PPM1M* и *PRICKLE2*.

Ключевые слова: светлоклеточная карцинома почки, гетерозиготные делеции, количественный ПЦР, анализ количества копий гена.

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