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Upregulation of *Rarb, Rarg*, and *Rorc* Genes in Clear Cell Renal Cell Carcinoma

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ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is one of the most frequent urological malignancies with a high mortality rate. The search for genes that could be potential targets for therapy as well as diagnostic and prognostic markers is an important task. In this work the expression of nine genes, encoding nuclear retinoid receptors, in ccRCC was analyzed by bioinformatics and quantitative PCR methods. We have identified the upregulation of *RARB, RARG*, and *RORC* genes that encode RAR-beta, RAR-gamma, and ROR-gamma nuclear retinoid receptors, correspondingly. As is well known, all of them can bind *trans*-retinoic acid. It allows assuming that the nuclear retinoid receptors may play significant role in the alterations of retinoic acid metabolism in ccRCC and contribute to the diseases progression.

Keywords: ccRCC, Rarb, Rarg, and Rorc Genes.

INTRODUCTION

Clear cell renal cancer (ccRCC) is the major histological type of kidney cancer and is characterized by high mortality, while morbidity has been steadily increasing in the world[1]. In most cases the ccRCC is sporadic, however, approximately 4% of cases had the hereditary reasons. Identification of the molecular mechanisms underlying the occurrence and development of kidney cancer, will not only make an important contribution to basic cancer, but also will serve as a basis for identification of potential targets for targeted therapy as well as diagnostic and prognostic markers.

It is shown that the violation of the metabolism of retinoids plays an important role in carcinogenesis. Retinoic acid and retinal are involved in signal transmission in cells, binding to nuclear receptors of retinoids. Their ligand is trans-retinoic acid. In mammals were identified several genes encoding retinoic acid receptor: the RARA gene encoding RAR-alpha; the RARB gene encoding RAR-beta and RARG gene encoding RAR-gamma[43].

For X retinoids receptors a ligand was 9-cis-retinoic acid. Were identified several genes encoding receptors of X retinoids: gene RXRA is encoding RÕR-alpha; gene RXRB is encoding RÕR-beta and gene RXRG is encoding RÕR-gamma.

For the so-called “orphan-receptors” of retinoids, at the moment there are some several known ligands: melatonin, CGP 52608, cholesterol and its derivatives, as well as a number of some synthetic compounds[24; 25; 36; 49; 50; 56; 58]. There is an evidence of a fully binding of transe-retinoic acid with ROR-beta, as an antagonist of transactivation function of the receptor[52]. The subfamily of orphan receptors of retinoids includes the products of three genes: RORA gene encoding ROR-alpha; the RORB gene encoding ROR-beta, and RORC gene encoding ROR-gamma.

In tumor cells the metabolic products of retinol may have an impact on their growth and differentiation by activating nuclear receptors of retinoids. Retinoic acid is associatedwith greater affinity with the retinoic acid receptor comparing with receptors of X retinoids[21]. In addition, there is the evidence that antiproliferative activity of retinoic acid is mediated through RAR-beta, and not through the RAR-alpha or RAR-gamma. It is shown that the expression of RAR-beta is reduced in the cell lines of tumors of the human kidney that are resistant to retinoic acid[4]. Retinoic acid may also bind with heterodimers, such as RAR-RXR.

The mechanism of action of retinoic acid on differentiation process in a number of cancers, differs from the mechanism of action of cytotoxic drugs used in modern medicine. These drugs mainly eliminate tumor cells, thereby reducing the proliferative potential of the tumor. Retinoic acid promotes differentiation and reduces the proliferation of tumor cells[47].

Today is studied a possibility of a combined treatment of malignancies with a use of retinoic acid and its derivate in a complex with other preparations, in particular, a molecular mechanisms of their interaction and a patient‘s response dynamics[30; 33; 15; 35; 58].

Binding to nuclear receptors is the latest stage in the transformation of retinoids for occurrence of effects associated with their effects on cells. Violation of this phase may be a key process responsible for the implementation by retinoids for their cellular functions. Therefore, to evaluate the involvement of the genes encoding the receptors of retinoids in the pathogenesis of ccRCC was undertaken quantitative analysis of their expression at the mRNA level.

MATERIAL AND METHODS

Bioinformatics Analysis

We analyzed TCGA RNA-Seq datasets (read counts) for kidney cancers using CrossHub software[28]. Using CrossHub, we have profiled expression levels of these transcripts in both paired and pooled samples and analyzed associations with disease stage, follow-up status, TNM indexes.

Tissue specimens

A total of 40 paired specimens of stages I–III of clear-cell renal-cell carcinoma and adjacent morphologically normal tissues were taken during surgical resection from patients with primary ccRCC without neoadjuvant therapy. Samples were frozen and stored in liquid nitrogen immediately after surgery. The diagnosis was verified by histopathology and only samples containing 70–80% or more tumor cells were used in the study. The tissue samples were collected in accordance with the guidelines issued by the Ethics Committee in National Medical Research Radiological Center, the Ministry of Health of the Russian Federation. All patients gave written informed consent, which is available upon request. The study was carried out in accordance with the principles outlined in the Declaration of Helsinki (1964).

The allocation of RNA and obtaining of cDNA

Frozen in liquid nitrogen samples of tumor and normal kidney tissue were subjected to mechanical homogenization on microdismembrator S Sartorius (Germany). For allocation of RNA was used the kit “RNeasy Mini Kit” (Qiagen, the Netherlands). Evaluation of the quantity of RNA was carried out on the NanoDrop 1000 spectrophotometer, (Nanodrop, USA) and on fluorimeter Qubit 2.0 (Invitrogen, USA), an assessment of quality – on the quality analyzer of nucleic acids the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RIN parameter (RNA Integrity Number, a quality score RNA) for the samples used in further studies, was at least 7. The allocated RNA was treated with Dnasa I (Thermo Fisher Scientific, USA). For the reaction of reverse transcription on

the matrix of mRNA was used revertase M-MuLV (Thermo Fisher Scientific, USA) and a set of random hexanucleotides.

Quantitative PCR (qPCR)

TaqMan Gene Expression Assays (Thermo Fisher Scientific, USA) were used to estimate the mRNA level of genes. The following kits were selected: *RARA* (Hs00940446_m1); *RARB* (Hs00977140_m1), *RARG* (Hs01559234_m1), *RXRA* (Hs01067640_m1), *RXRB* (Hs00232774_m1), *RORA* (Hs00536545_m1) and *RORC* (Hs01076112_m1). Genes *RPN1* and *GUSB* were used as a reference as described earlier [27, 40, 41]. All reactions were performed using AB 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). PCR program was as follows: 10min at 95°C and then 50 two-step cycles 15 s at 95°C and 60 s at 60°C. Reactions were performed in triplicate. The Relative Quantitation software (Thermo Fisher Scientific, USA) and ATG (Analysis of Transcription of Genes) tool were used to analyze the obtained qPCR data as described earlier[34]. The inner variability between mRNA levels of reference genes do not exceed 2 times, and therefore 2-fold or more expression alterations of the target genes were considered significant. Inter-group and intra-group comparisons were performed using nonparametric Wilcoxon/Mann-Whitney U–test and Kruskal-Wallis test. Differences with $p < 0.05$ were considered statistically significant. The statistical procedures were performed with BioStat software (AnalystSoft Inc., USA).

RESULTS

Bioinformatics analysis allowed a preliminary evaluation of the mRNA content of genes encoding receptors of retinoids in the tumor tissues of the kidney, and to exclude from the pilot study, genes with relatively low expression level - *RXRG*, and *RORB*. Results of quantitative assessment of gene expression are summarized in table 1.

Genes *RARA, RARB* and *RARG (RAR* family). In most of the samples (66%) the *RARA* gene expression did not change. In 29% of cases there was an increase in its expression up to 16-fold. A frequent increase in expression was observed in samples of predominantly II and III stages (60% and 67% of cases, respectively). In one sample was revealed a slight decrease in the expression of *RARA* gene in 2 times. The mean value of relative mRNA level for the whole sample was 1.6.

In 55% of samples of ccRCC was shown an increased expression of *RARB* gene in 2 - 22 times. In two samples there was a slight decrease in its expression in 2 - 3 times. In 35% of cases the expression of RARB gene was not changed. The

Table 1: The frequency and extent of changes in the relative level of mRNA of the genes encoding the retinoic acid receptor, in ccRCC.

Gene	Clear cell renal cell carcinoma		
	The average change	The frequency of events	
		↓	↑
RARA	1,6↑	5% (2-fold)	29% (2 - 16)
RARB	2,2↑	10% (3-fold)	55% (2 - 22)
RARG	1,9↑	5% (2.5-fold)	49% (2 - 52)
RXRA	1,4↑	5% (2.5-fold)	24% (2 - 14)
RXRB	1	19% (2 - 5)	19% (2 - 9)
RORA	1,3↑	16% (2 - 3)	21% (3 - 21,5)
RORÑ	1,9↑	21% (2 - 7)	46% (2 - 35)

Note: ↓ – decreased level of mRNA, ↑ – increased level of mRNA. An average change was calculated as the geometric mean of relative mRNA levels. qPCR data.

mean value of relative mRNA level for the entire sample was 2.2. A characteristic feature of the profile of mRNA of this gene – an increased expression mainly at the first stage. As the progression of the disease the expression is normalizing, and in samples of the III stage is not changed in comparison with surrounding conditionally normal tissue.

In 49% of samples of ccRCC was shown an increased RARG gene expression in 2 - 52 times. In two samples there was a slight decrease in the level of mRNA of the RARG gene in 2.5 times. In 46% of cases its expression did not change. The mean value of relative mRNA level for the entire sample was 1.9. In the samples of the I-st stage the expression was increased in 63% of cases, in the samples of the II-nd stage the expression was increased in 50% of cases, in the third stage the expression was increased only in 12.5% of cases.

Genes RXRA and RXRB (RXR family). In most of the samples of ccRCC (72%) the expression of RARA gene did not change. In 24% of cases was shown an increased expression in 2 - 14 times. In one sample was observed a slightly decrease in expression of RARA gene in 2.5 times. The mean value of relative mRNA level for the entire sample was 1.4.

In 19% of samples of ccRCC was shown an increased RXRB gene expression in 2-8 times. In 19% there was a decrease in its expression in 2-5 times. In 62% of studied samples the expression of RXRB gene was not changed. The mean value of relative mRNA level for the entire sample was 1,0.

RORA and *RORC* genes (ROR family). In most of the samples of ccRCC (63%) the expression of RORA gene was not altered. In 21% of cases was shown an increase of expression in 3 - 21.5 times. In 16% of samples was observed a decrease in the expression of RORA gene in 2 - 3 times. The mean value of relative mRNA level for the entire sample was 1.3.

In 46% of samples of ccRCC was observed an increase in *RORC* gene expression in 2 - 35 times. In 20.5% of cases was observed a reduction in its expression in 2 - 7 times. In 33% of tested samples the expression of *RORC* gene did not change. The mean value of relative mRNA level for the entire sample was 1.9. A characteristic feature of *RORC* gene - a significant increase in expression predominantly in samples of the II-nd stage.

DISCUSSION

Genes RARA, RARB and RARG (family RAR). The retinoic acid receptor can regulate the expression of many genes[7]. In the modern biomedical literature there are many works which studied the involvement of RARA, RARB and RARG genes in oncogenesis. Thus, in most human carcinoma were revealed either a significant increase in the gene expression of RARA (hepatocellular carcinoma, adenocarcinoma of the esophagus, non-small cell lung cancer)[44; 32;48], and either a significant decrease (stomach cancer, cell line carcinoma of the cervix)[14;22]. It is shown that the decrease of RARA gene expression in cancer of the cervix, esophagus, colorectal cancer, neuroblastoma and breast cancer is mainly its hypermethylation of a promoter region[3;5;10;11;29;45]. In prostate cancer was revealed a correlation of RARA gene expression with the degree of a tumor differentiation[16]. In a number of studies it was shown that the protein RAR-alpha regulates differentiation of tumor cells in leukemia, breast cancer, cancer of the head and neck and teratocarcinoma[12;51;53;56;59].

Was shown a violation of RARB gene expression in most cases of cancer of the lung, esophagus, breast, pancreas and cervical cancers[60]. It is established that in most cases of cancer of the pancreas, prostate, bladder and lung cancer the RARB gene is methylated[8;13;17;18;19;38;39;55]. It is shown that the RARG gene expression is reduced in NSCLC, adenocarcinoma of the esophagus and increases in HCC[32;42;44]. Reliable data about gene expression of RARA, RARB and RARG in kidney tumors do not exist. For the first time we have shown the violation of the expression of these three genes that may be important in the onset and progression of ccRCC.

Genes RXRA and RXRB (RXR family). In most types of human carcinoma was shown a disruption of the expression of genes encoding receptors of X retinoids. Thus, it was revealed an

increase in the expression of *RXRA* gene in laryngeal cancer, and breast cancer. At non-small cell lung cancer, stomach cancer and thyroid cancer was shown a decrease in the expression of *RXRA* gene[6;54;20]. For RXRB gene in a number of studies was shown a reduced expression in cancer of the head and neck, gastric cancer, prostate, thyroid and non-small cell lung cancer[6;2;20;33;46;54]. In patients with ovarian cancer at a late stage is revealed an overexpression of RXRB gene[23]. In the modern literature there are no data about the RXRA gene expression and RXRB in kidney tumors. It is shown that the expression of *RXRA* gene is increased in about a quarter of the samples, while the increase and decrease of *RXRA* expression occur with the same frequency. However, the data demonstrate the involvement of genes of receptors family of X retinoids in carcinogenesis in cRCC and there is a deregulation of their expression. Perhaps the level of expression of these genes is related to certain characteristics of the tumor.

Genes *RORA and RORC* (*ROR family*). In the modern literature are presented several works on the involvement of RORA gene in carcinogenesis. It is shown that the increasing of RORA gene expression can inhibit the proliferation of neoplastic prostate cells[37]. In addition, it was shown the violations of *RORA* expression in diffuse gastric cancer, kidney and colon[26]. In colorectal cancer it was shown that the protein ROR-alpha is involved in Wnt-signaling pathways[31]. In cell lines of hepatoma (HepG2) were revealed changes in the expression of target genes of the transcription factor ROR-alpha[9]. Data about the expression *of RORC* gene in the contemporary literature are absent. We identified a multi-directional change in gene expression which demonstrates that the ROR family genes have also been subjected to deregulation of gene expression.

Thus, for the first time it was performed a quantitative expression analysis of genes encoding nuclear receptors of retinoids in clear cell renal cell carcinoma in humans. The study with a method of qPCR allowed us to identify genes with the deregulation of expression and genes with increased expression primarily. The obtained data suggest that in cells with ccCC-RCC, naturally occurring dysregulation of the metabolism of retinol. Apparently, in tumor cells there is an impairment, mainly at the stages of formation of the products of metabolism of retinol – retinoic aldehydes and acids, which are the main ligands of receptors of retinoids. In this regard, the nuclear receptors of retinoids are not able to perform its function (activation or inhibition of transcription).

CONCLUSION

We first identified the upregulation of *RARB, RARG*, and *RORC* genes in ccRCC. The obtained results allow assuming that the nuclear retinoid receptors RAR-beta, RAR-gamma and ROR-gamma, which bind *trans*-retinoic acid, may play an important role in the alterations of retinoic acid metabolism in ccRCC and contribute to the diseases progression.

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