Deregulation of *RBP4* and *RBP7* Genes in Clear Cell Renal Cell Carcinoma

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Abstract

Background: The incidence of clear cell renal cell carcinoma (ccRCC) steadily increases each year, while the molecular mechanisms involved in the initiation and progression of the disease remain unclear. Therefore, 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, we studied the expression of genes, encoding retinol binding proteins, in ccRCC. **Materials and Methods:** Using quantitative polymerase chain reaction, we analyzed the expression of *RBP2*, *RBP4*, and *RBP7* genes in a representative set of ccRCC samples. **Results:** We identified deregulation of *RPB4* and *RBP7* genes in ccRCC. Significant decrease in the expression of the *RBP4* gene was shown in most ccRCC samples. In contrast, the increase in mRNA level of the *RBP7* gene was observed in almost all cases. **Conclusions:** Obtained results allow us to assume that *RBP4* and *RBP7* could be involved in ccRCC carcinogenesis. Moreover, our data confirm alterations in retinoid metabolism in ccRCC.

Key words: Clear cell renal cell carcinoma, gene expression, qPCR, retinol binding proteins, retinol metabolism

INTRODUCTION

Renal cancers account for approximately 2-3% of all malignancies in adults, and renal cell carcinoma (RCC) is diagnosed in 80-90% of the cases. However, the origin and evolution of RCC are poorly understood. Most RCCs are sporadic, but around 2-4% of cases result from familial susceptibility. The disease is almost two times more common in men than in women. Clear cell RCC (ccRCC) represents the largest subgroup of RCC and has the highest rates of local invasion, metastasis, and mortality.[1,2] It is known that ccRCC is a metabolic disorder. Numerous genetic and epigenetic changes, occurring in ccRCC, effect transcription of many genes and lead to alterations in different metabolic pathways.[3-8] Deregulation of metabolic pathways in ccRCC determines its aggressive phenotype.[9] Understanding the molecular mechanisms leading to altered metabolic pathways in ccRCC could provide novel targets for therapies and diagnostic biomarkers.

Vitamin A (retinol) and its derivatives play an important role in many biological processes and influence cell differentiation, proliferation, and apoptosis.[10] A number of studies have suggested an association between development of cancer and dietary intake of total vitamin A.[11] Retinoids were shown to be active in the treatment of preneoplastic diseases[12,13] and different malignancies, including breast, lung, skin, kidney, hepatic, and other cancers.[14-19] These provide a basis for the use of retinoids in cancer treatment and chemoprevention.

In this work, we have analyzed the expression of several genes encoding retinol binding proteins in ccRCC using quantitative polymerase chain reaction (qPCR). Deregulation in the expression of *RBP4* and *RBP7* genes was revealed.

Obtained results may indicate the alterations in retinoid metabolism during ccRCC carcinogenesis and involvement of *RBP4* and *RPB7* genes in the process.

MATERIALS AND METHODS

Tissue samples

A total of 38 samples of ccRCC (I-IV Stages) and adjacent morphologically normal tissues (conventional "normal" tissues) were obtained after surgical resection before radiation or chemotherapy. The samples were frozen immediately after surgery and stored in liquid nitrogen. The diagnosis was verified by histopathology, and only samples containing 70-80% or more tumor cells were used in the study. Each patient provided written consent and gave permission for the use of their samples for research purposes. The study was approved by the Ethics Committee of Herzen Moscow Cancer Research Institute, the Ministry of Health of the Russian Federation. The study was conducted in accordance with the principles outlined in the Declaration of Helsinki (1964).

RNA isolation and cDNA synthesis

RNA was isolated from the samples using mikro dismembrator S (Sartorius, Germany) and RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocols. RNA was quantified on Nanodrop spectrophotometer (Nanodrop Tech., USA). The Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer system (Agilent Tech., USA) were used to determine the RNA integrity number (RIN). The isolated RNA was treated with DNAsa I (Thermo Fisher Scientific, USA) according to manufacturer's instructions. cDNA synthesis was performed using M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, USA) and random hexamers according to the standard manufacturer's protocol.

qPCR and statistical analysis

QPCR reactions were carried out on applied biosystems 7500 real-time PCR System (Thermo Fisher Scientific, USA). TaqMan Gene Expression Assays (*RBP2*: Hs00188160_m1, *RBP4*: Hs00198830_m1, *RBP7*: Hs00364812_m1) (Thermo Fisher Scientific, USA) were used to estimate the mRNA level of target genes. qPCR procedure was performed as described.[20] PCR products were analyzed in 2% agarose gels, purified and submitted for Sanger sequencing on an ABI Prism 3100 Genetic Analyzer (Thermo Fisher Scientific, USA). qPCR data were analyzed using two reference genes, *GUSB* and *RPN1*,[21,22] and ΔΔCt-method with ATG program (Analysis of Transcription of Genes).[23-25] Each reaction was repeated three times. At least 2-fold mRNA changes were considered as significant because of reference genes variability.

Nonparametric Wilcoxon test was used to compare mRNA expression differences of target and reference genes in ccRCC samples. $P < 0.05$ was considered statistically significant.

RESULTS

Down-regulation of *RBP4*

We revealed more than 100-fold down-regulation of the *RBP4* gene in 87% (33 of 37, $P < 0.05$) of ccRCC samples [Figure 1]. Decreased expression of the *RBP4* gene was particularly observed in ccRCC of stages II and III. The mRNA level of *RBP4* was increased in two cases by 3- and 20-fold. The mean value of relative mRNA level decrease was 32.6.

Up-regulation of *RBP7*

A significant increase in the mRNA level of the *RBP7* gene was observed in 79% (30 of 38, $P < 0.05$) of examined ccRCC samples [Figure 2]. Up-regulation of *RBP7* was mainly found in early-stage ccRCC. Decreased expression of *RBP7* was detected in three cases (from 4- to 8-fold). The mean value of relative mRNA level increase was 15.4.

The mRNA level of *RBP2* gene was very low and could not be detected by qPCR method.

DISCUSSION

Vitamin A is obtained from a diet in the form of retinyl esters (animal sources) or carotenoids (plant food). In the intestine, retinyl esters/carotenoids are converted to retinol, which then is secreted through the lymphatic system into the blood and is taken up by hepatocytes. The liver stores the retinol and provides it to the body. Retinol is secreted from the liver into the blood and bound to plasma RBP4. Although the liver is the main site of RBP4 synthesis, the protein can be also expressed and presumably secreted by other tissues, such as adipose, lung, kidney, testis, brain, and retinal pigment epithelium in the eye.[26] Cellular RBPs (RBP2, RBP5, and RBP7) play a role in the cellular metabolism of retinol and vitamin A stability. For example, the vitamin A metabolite, retinoic acid, can regulate the transcription of numerous genes, and its effect is mediated by cellular retinoid binding proteins.[27-29]

Recent studies have reported that RBPs may be involved in the development of cancer. The plasma level of RBP4 was shown to be associated with risk of colon adenoma,[30] and the increased serum level of RBP4 was detected in pancreatic cancer patients.[31] It has been suggested that the decrease in the serum level of RBP4 might be a potential biomarker of ovarian cancer.[32] Frequent methylation of the *RBP4* gene
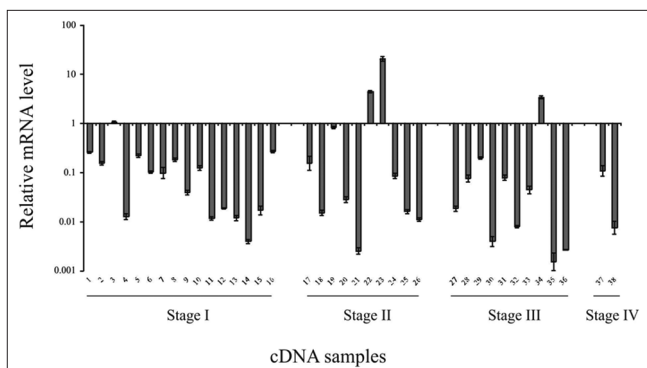
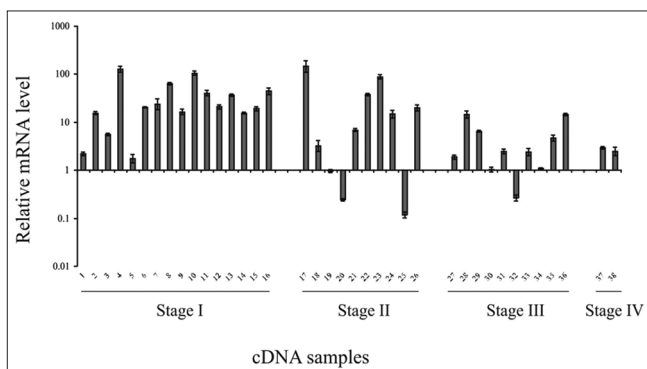**Figure 1:** Relative mRNA level of *RBP4* gene in ccRCC. QPCR data

Figure 2: Relative mRNA level of *RBP7* gene in ccRCC. QPCRdata

was shown in esophageal squamous cell carcinoma.[33] In addition, other RBPs have been studied to a lesser extent, and their participation in cancer was not shown.

Using qPCR, we first revealed deregulation of *RBP4* and *RBP7* genes in ccRCC. A decreased mRNA level of the *RBP4* gene was demonstrated in the examined samples, in contrast, a significant increase in *RBP7* expression was shown in most ccRCC cases. These data indicate that alterations of retinoid metabolism, effecting both intra- and extracellular transport of retinol, may be associated with the development of cancer. Moreover, the findings implicate *RBP4* and *RBP7* expression levels and their correlation with the disease stage as potential diagnostic or prognostic biomarkers of ccRCC.

CONCLUSION

Our study revealed a significant deregulation of *RBP4* and *RBP7* gene expression in ccRCC. These proteins play an important role in retinoid metabolism. Extracellularly and intracellularly, they bind to retinol, solubilize and stabilize it, and then transport retinol from retinoid stores to target tissues. Thus, the obtained results allow us to assume that alterations in the regulation of the transport, metabolism, and action of retinol may occur in ccRCC carcinogenesis.

REFERENCES

1. Amin MB, Amin MB, Tamboli P, Javidan J, Stricker H, de-Peralta Venturina M, *et al.* Prognostic impact of histologic subtyping of adult renal epithelial neoplasms: An experience of 405 cases. Am J Surg Pathol 2002;26:281-91.
2. Moch H, Gasser T, Amin MB, Torhorst J, Sauter G, Mihatsch MJ. Prognostic utility of the recently recommended histologic classification and revised TNM staging system of renal cell carcinoma: A Swiss experience with 588 tumors. Cancer 2000;89:604-14.
3. Dmitriev AA, Rudenko EE, Kudryavtseva AV, Krasnov GS, Gordiyuk VV, Melnikova NV, *et al.* Epigenetic alterations of chromosome 3 revealed by NotI-microarrays in clear cell renal cell carcinoma. Biomed Res Int 2014;2014:735292.
4. Oparina NY, Snezhkina AV, Sadritdinova AF, Veselovskii VA, Dmitriev AA, Senchenko VN, *et al.* Differential expression of genes that encode glycolysis enzymes in kidney and lung cancer in humans. Genetika 2013;49:814-23.
5. Loginov VI, Dmitriev AA, Senchenko VN, Pronina IV, Khodyrev DS, Kudryavtseva AV, *et al.* Tumor suppressor function of the SEMA3B gene in human lung and renal cancers. PLoS One 2015;10:e0123369.
6. Kudriavtseva AV, Anedchenko EA, Oparina NIu, Krasnov GS, Kashkin KN, Dmitriev AA, *et al.* Expression of FTL and FTH genes encoding ferretin subunits in lung and renal carcinomas. Mol Biol (Mosk) 2009;43:1044-54.
7. Braga EA, Khodyrev DS, Loginov VI, Pronina IV, Senchenko VN, Dmitriev AA, *et al.* Methylation in the regulation of the expression of chromosome 3 and microRNA genes in clear-cell renal cell carcinomas. Genetika 2015;51:668-84.
8. Oparina NY, Sadritdinova AF, Snezhkina AV, Dmitriev AA, Krasnov GS, Senchenko VN, *et al.* Increase in NETO2 gene expression is a potential molecular genetic marker in renal and lung cancers. Genetika 2012;48:599-607.
9. Zaravinos A, Pieri M, Mourmouras N, Anastasiadou N, Zouvani I, Delakas D, *et al.* Altered metabolic pathways in clear cell renal cell carcinoma: A meta-analysis and validation study focused on the deregulated genes and

their associated networks. Oncoscience 2014;1:117-31.

10. Blomhoff R, Green MH, Green JB, Berg T, Norum KR. Vitamin A metabolism: New perspectives on absorption, transport, and storage. Physiol Rev 1991;71:951-90.

11. Doldo E, Costanza G, Agostinelli S, Tarquini C, Ferlosio A, Arcuri G, *et al.* Vitamin A, cancer treatment and prevention: The new role of cellular retinol binding proteins. Biomed Res Int 2015;2015:624627.

12. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science 1997;278:1073-7.

13. Sun SY, Lotan R. Retinoids and their receptors in cancer development and chemoprevention. Crit Rev Oncol Hematol 2002;41:41-55.

14. Bryan M, Pulte ED, Toomey KC, Pliner L, Pavlick AC, Saunders T, *et al.* A pilot phase II trial of all-trans retinoic acid (Vesanoid) and paclitaxel (Taxol) in patients with recurrent or metastatic breast cancer. Invest New Drugs 2011;29:1482-7.

15. Arrieta O, González-De la Rosa CH, Aréchaga-Ocampo E, Villanueva-Rodríguez G, Cerón-Lizárraga TL, Martínez-Barrera L, *et al.* Randomized phase II trial of All-trans-retinoic acid with chemotherapy based on paclitaxel and cisplatin as first-line treatment in patients with advanced non-small-cell lung cancer. J Clin Oncol 2010;28:3463-71.

16. Moore DM, Kalvakolanu DV, Lippman SM, Kavanagh JJ, Hong WK, Borden EC, *et al.* Retinoic acid and interferon in human cancer: Mechanistic and clinical studies. Semin Hematol 1994;31 4 Suppl 5:31-7.

17. Berg WJ, Nanus DM, Leung A, Brown KT, Hutchinson B, Mazumdar M, *et al.* Up-regulation of retinoic acid receptor beta expression in renal cancers *in vivo* correlates with response to 13-cis-retinoic acid and interferon-alpha-2a. Clin Cancer Res 1999;5:1671-5.

18. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nat Rev Cancer 2001;1:181-93.

19. Siddikuzzaman, Guruvayoorappan C, Berlin Grace VM. All trans retinoic acid and cancer. Immunopharmacol Immunotoxicol 2011;33:241-9.

20. Snezhkina AV, Krasnov GS, Lipatova AV, Sadritdinova AF, Kardymon OL, Fedorova MS, *et al.* The dysregulation of polyamine metabolism in colorectal cancer is associated with overexpression of c-Myc and C/EBPß rather than enterotoxigenic bacteroides fragilis infection. Oxid Med Cell Longev 2016;2016:2353560.

21. Krasnov GS, Oparina NIu, Dmitriev AA, Kudriavtsev AV, Anedchenko EA, Kondrat'eva TT, *et al.* Novel reference gene RPN1 for normalization of quantitative data in lung and kidney cancer. Mol Biol (Mosk) 2011;45:238-48.

22. Fedorova MS, Kudryavtseva AV, Lakunina VA, Snezhkina AV, Volchenko NN, Slavnova EN, *et al.* Downregulation of OGDHL expression is associated with promoter hypermethylation in colorectal cancer. Mol Biol (Mosk) 2015;49:678-88.

23. Dmitriev AA, Kudryavtseva AV, Krasnov GS, Koroban NV, Speranskaya AS, Krinitsina, *et al.* Gene expression profiling of flax (*Linum usitatissimum* L.) under edaphic stress. BMC Plant Biol 2016;16 Suppl 3:237.

24. Melnikova NV, Dmitriev AA, Belenikin MS, Koroban NV, Speranskaya AS, Krinitsina AA, *et al.* Identification, expression analysis, and target prediction of flax genotroph micrornas under normal and nutrient stress conditions. Front Plant Sci 2016;7:399.

25. Dmitriev AA, Krasnov GS, Rozhmina TA, Kishlyan NV, Zyablitsin AV, Sadritdinova AF, *et al.* Glutathione S-transferases and UDP-glycosyltransferases are involved in response to aluminum stress in flax. Front Plant Sci 2016;7:1920.

26. Noy N, Li L, Abola MV, Berger NA. Is retinol binding protein 4 a link between adiposity and cancer? Horm Mol Biol Clin Investig 2015;23:39-46.

27. Newcomer ME. Retinoid-binding proteins: Structural determinants important for function. FASEB J 1995;9:229-39.

28. Napoli JL. Retinoic acid biosynthesis and metabolism. FASEB J Off Publ Fede Am Soc Exp Biol 1996;10:993-1001.

29. Kwong J, Lo KW, Chow LS, To KF, Choy KW, Chan FL, *et al.* Epigenetic silencing of cellular retinol-binding proteins in nasopharyngeal carcinoma. Neoplasia 2005;7:67-74.

30. Abola MV, Thompson CL, Chen Z, Chak A, Berger NA, Kirwan JP, *et al.* Serum levels of retinol-binding protein 4 and risk of colon adenoma. Endocr Relat Cancer 2015;22:L1-4.

31. El-Mesallamy HO, Hamdy NM, Zaghloul AS, Sallam AM. Serum retinol binding protein-4 and neutrophil gelatinase-associated lipocalin are interrelated in pancreatic cancer patients. Scand J Clin Lab Invest 2012;72:602-7.

32. Lorkova L, Pospisilova J, Lacheta J, Leahomschi S, Zivny J, Cibula D, *et al.* Decreased concentrations of retinol-binding protein 4 in sera of epithelial ovarian cancer patients: A potential biomarker identified by proteomics. Oncol Rep 2012;27:318-24.

33. Tsunoda S, Smith E, De Young NJ, Wang X, Tian ZQ, Liu JF, *et al.* Methylation of CLDN6, FBN2, RBP1, RBP4, TFPI2, and TMEFF2 in esophageal squamous cell carcinoma. Oncol Rep 2009;21:1067-73.