# Clear cytoplasm of renal cell carcinoma is more than a morphologic phenotype: relation to drug sensitivity, AMPK/PGC-1α pathway, and mitochondria

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# Clear cytoplasm of renal cell carcinoma is more than a morphologic phenotype: relation to drug sensitivity, AMPK/PGC-1α pathway, and mitochondria

Directed by Professor Woong Kyu Han

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This certifies that the Doctoral Dissertation of Joon Chae Na is approved.

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# ABSTRACT

Clear cytoplasm of renal cell carcinoma is more than a morphologic phenotype: relation to drug sensitivity, AMPK/PGC-1α pathway, and mitochondria

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(Directed by Professor Woong Kyu Han)

Clear cytoplasm is a distinct histologic feature of clear cell renal cell carcinoma (ccRCC), the most prevalent kidney cancer. The pathophysiologic significance of clear cytoplasm has not been previously studied; thus, this study aimed to unveil the role of clear cytoplasm. Clear cytoplasm induction (CCI) of renal cancer cell lines was performed by incubation in adipogenic induction medium. Response to temsirolimus was enhanced, mitochondrial biogenesis was increased, and genes related to adipogenesis were differentially regulated after CCI in ccRCC cells. Genes differentially regulated after CCI were screened in a microarray dataset of ccRCC tissues. The expression of *PPARGC1A*, *SFRP1*, and *GATA2* was decreased in tumor tissue compared to that in normal kidney tissue; these genes were upregulated after CCI. Expression of *TWIST1* in

Caki-1 cells was higher in tumor tissue than that in normal kidney tissue and downregulated after CCI. Among the screened genes, *PPARGC1A* was further studied as it encodes PGC-1α, a regulator of mitochondria and lipogenesis. The AMPK-PGC-1α pathway was found to be activated after CCI. In conclusion, the presence of clear cytoplasm affects the therapeutic response of RCC cells, and the mitochondrial activity and AMPK/PGC-1α pathway are affected by CCI

Keywords: kidney cancer, clear cell renal cell carcinoma, clear cytoplasm, adipogenesis, mitochondria, peroxisome proliferator-activated receptor gamma coactivator 1-alpha

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## I. INTRODUCTION

Renal cell carcinoma (RCC) is thought to result from dysregulated cellular metabolism: known mutations in genes causing RCC, such as von Hippel-Lindau tumor suppressor (*VHL*), mitochondrial fumarate hydratase (*FH*), and succinate dehydrogenases (*SDH*; *SDHB*, *SDHC*, *SDHD*), lead to the dysregulation of at least one metabolic pathway mediated by oxygen, iron, energy, or nutrient sensing. [1] Among the various cancers originating in the kidney, clear-cell RCC (ccRCC) is the most common subtype (83%) and has a distinct cellular morphology: a clear cytoplasm, from which the name “clear cell” originates. (Fig1A) The transparency of the cytoplasm results from accumulation of glycogens and lipids. [2] This clear cell morphology is lost in ccRCC cell lines; ccRCC cells no longer have easily identifiable morphological characteristics used in the histological classification of RCC specimens when the cells are immortalized and grown in general culture medium used for *in vitro* studies. [3] This is probably due to changes in the microenvironment and the deprivation of interactions with surrounding tissue cells. Loss of this distinct

morphological feature in ccRCC cell lines suggests a change in the metabolism of lipids or glycogens, and considering the significant role of metabolic dysregulation in RCC, this change may affect tumor characteristics. However, the effect of induction of clear cytoplasm, or loss of it, on the characteristics of RCC cells has not been studied. Here, we investigated the effect of clear cytoplasm induction (CCI) on RCC cells and the intracellular changes caused by CCI.

Mitochondria are the major energy and metabolite source in the cell, and their dysregulation, as it relates to cancer, is gaining interest as key oncogenes and tumor suppressors are identified as modulators of mitochondrial metabolism and dynamics. [4] RCC is thought to be metabolically reprogrammed toward low mitochondrial oxidative phosphorylation, which results from decreases in mitochondrial DNA, respiratory chain activities, and oxidative phosphorylation- related proteins. Our group and others have shown that proteins related to mitochondria, such as Sirt3 and PGC-1α, are related to the carcinogenesis of RCC. [5-7] To further expand on this work, we herein investigated the effect of CCI on mitochondria.

In this study, we induced clear cytoplasm in RCC cell lines by adipogenic induction and found that restoring clear cytoplasm in RCC cells increases the sensitivity of RCC cells to temsirolimus, a target therapeutic agent. We studied changes on mitochondria by CCI and found that mitochondrial biogenesis was increased and that multiple genes related to adipogenesis were differentially regulated. These genes were cross-screened with our microarray dataset of ccRCC patients, and *PPARGC1A* was identified as a promising candidate that affects tumor characteristics of ccRCC cells. Further investigation confirmed the activation of the AMPK/PGC-1α pathway.

## II. MATERIALS AND METHODS

### 1. Cell culture and adipogenic differentiation

Human kidney carcinoma cell lines Caki-1 and Caki-2 were obtained from ATCC. Cell lines were authenticated and tested for mycoplasma contamination. The cell lines were cultured in McCoy's medium (Gibco, Grand Island, NY, US) containing 10% fetal bovine serum and 1% penicillin/streptomycin under an atmosphere of 5% CO2 and 95% O2 at 37°C. The cell lines were subcultured every 7 days using 0.02% ethylenediaminetetraacetic acid and 0.05% trypsin. The medium was replaced with fresh medium every 2 days. For CCI, the medium was replaced with adipogenic induction medium (Lonza Bioscience, Verviers, Belgium) containing recombinant human insulin, L-glutamine, mesenchymal stem cell growth supplement, penicillin/streptomycin, dexamethasone, indomethacin, and IBMX (3-isobutyl-1-methylxanthine). After one cycle of induction/maintenance, the cells were cultured for 3 days in adipogenic induction medium, which was replaced every 2–3 days.

2. Nile red staining

Cells were grown on an eight-well chamber Labtek slide (Nalgene, Rochester, NY), rinsed with ice-cold PBS, and fixed with 4% paraformaldehyde solution at 4°C for 10 min. After rinsing, the cells were stained with 0.2 μg/ml 9-diethylamino-5H-benzo[alpha]phenoxazine-5-one (Nile red), a selective fluorescent dye for intracellular lipid droplets (Sigma-Aldrich, St. Louis, MO), at room temperature for 10 min. We removed excess dye by briefly rinsing with PBS. After rinsing, the slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Lipid vesicles were observed with excitation at 530 nm and emission at 635 nm using a confocal microscope (LSM Meta 700, Carl Zeiss, Oberkochen, Germany).

3. Cell toxicity assay (CCK-8 assay)

Cells in a 24-well culture plate were treated with different doses of

temsirolimus for 24 hr. The cell toxicity was detected using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). WST-8 (water-soluble tetrazolium salt; 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt) was added in each well, and cells were incubated for 1 hour. Water-soluble formazan dye, produced from WST-8 by reduction of dehydrogenases in cells, was directly proportional to the number of living cells. WST-8 was measured using a Beckman Coulter microplate reader at 450 nm.

4. RNA isolation and quantitative PCR

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. For cDNA synthesis, 500 ng of RNA from each sample was reverse transcribed using the RT2 First Strand kit (Qiagen). Human adipogenesis RT2 Profiler PCR Array and human mitochondria RT2 Profiler PCR Array (Qiagen) were carried out with RT2 SYBR green master mix using the Applied-Biosystem step one plus system (Applied-Biosystem, Foster city, CA). All procedures were performed according to the manufacturer's instructions. cDNA analyses were performed in duplicate for all analyses. Data were analyzed using the ΔΔCT method and normalized to RPLP0.

5. Immunofluorescence assay

The expression levels of the voltage-dependent anion channel (VDAC) and peroxisome proliferator-activated receptor ɤ coactivator-1α (PGC-1α) were compared via an immunofluorescence assay. Cells were cultured in an eight-well chamber cell slide (Labteck). Cells were fixed with 4% paraformaldehyde solution for 10 minutes and then washed with PBS. Cells were permeated in 0.5% Triton X-100/PBS for 5 min, blocked in 5% BSA/PBS for 1 hr, and incubated with diluted primary antibodies of anti-VDAC (Abcam)

and anti-PGC-1α (Abcam) in 5% BSA/PBS 4°C for 16 hr. The cells were then washed with PBS-T and treated with secondary antibodies tagged with Alexa Fluor 488 (Invitrogen) at 25°C for 1 hr (1:200). Images were taken with a confocal microscope (LSM Meta 700, Carl Zeiss, Oberkochen, Germany) and analyzed using LSM Image Browser software.

6. Mitochondrial membrane potential assay

Cells in a glass-bottom dish (glass diameter 10 mm) were treated with mitochondrial probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Cayman Chemical, Ann Arbor, MI, US) and incubated at 37°C for 20 min. Healthy cells with mainly JC-1 aggregates were detected with excitation at 540 nm and emission at 570 nm, whereas apoptotic or unhealthy cells with mainly JC-1 monomers were detected with excitation at 485 nm and emission at 535 nm with a confocal microscope (LSM Meta 700, Carl Zeiss, Oberkochen, Germany).

7. Western blot

Total cellular protein extract was prepared on ice using a PRO-PREP protein extract solution (Intron, Seoul, Korea). Cell lysates were loaded onto a sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidenefluoride membrane for 1 hr. The membrane was incubated overnight at 4°C with VDAC, PGC-1α, P-AMPK, and GAPDH primary antibodies (Abcam) diluted to 1:1000 with 5% BSA Tris-buffered saline-Tween 20 (TBS-T). Next, the membrane was washed with TBS-T and incubated with secondary antibodies (1:10000, horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG) at room temperature for 1 hr. Labeled bands were detected by using the West Pico chemiluminescent kit (Thermo scientific, Rockford, IL, US).

## III. RESULTS

### 1. CCI enhances response to temsirolimus

The typical histologic feature of ccRCC cells is lipid-laden cells resembling adipocytes; however, the clinical or pathophysiological significance of the abundant lipid droplets is unknown. RCC cell lines have been reported to have adipogenic differentiation potential, and the cells undergoing adipogenic differentiation achieve abundant clear cytoplasm, which resembles the clear cell appearance of ccRCC cells that are observed in tumor sample tissues. [8] We sought to explore how this morphologic change affects RCC cells.

Caki-1 (derived from metastasis to skin; *VHL* wild type) and Caki-2 (derived from primary kidney tumor; *VHL* loss-of-function mutation) cell lines were each cultured in adipogenic induction medium. Formation of lipid-like vacuoles was observed under direct visualization and confirmed as lipid droplets by Nile red staining. This adipogenic change was more prominent in Caki-1 cells than in Caki-2 cells. (Fig1B)

The adipogenesis-induced cells were then treated with temsirolimus. Native Caki-1 cells showed minimal response to temsirolimus; whereas, Caki-2 cells showed a moderate response. Response to the drug was clearly enhanced in cells with CCI when the dose was increased from 2.5 to 20 μM. Notably, Caki-1 cells, which were almost non-responsive to temsirolimus, were responsive to treatment after CCI. Viability of Caki-1 cells at 20 μM temsirolimus decreased from 92.3% to 75.0% after CCI. (Fig1C) The response of Caki-1 cells to sunitinib also increased after CCI (data not shown).

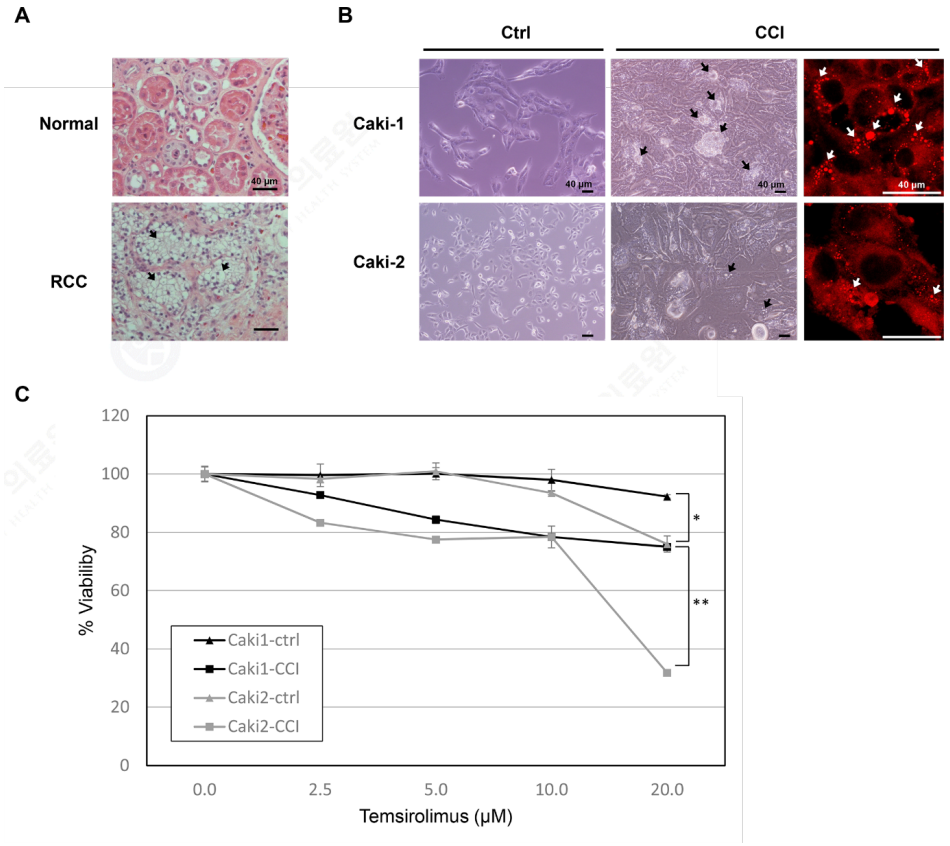


**Fig 1. CCI enhances response to temsirolimus.** A. Hematoxylin and eosin (H&E) stain of normal and ccRCC tissue samples. Abundant lipid cellular components of ccRCC were dissolved during de-paraffinization by xylene, which resulted in the "clear cytoplasm" appearance. B. Clear cytoplasm induction (CCI) of Caki-1 and Caki-2 cells. Caki-1 and Caki-2 cells were incubated in adipogenesis induction medium for 2 weeks. Left: before CCI, light microscopy; middle: after CCI, light microscopy; right: after CCI, Nile red staining. (Ctrl=control, before clear cytoplasm induction; CCI=after clear cytoplasm induction.) C. Response to temsirolimus of Caki-1 and Caki-2 cells, before and after CCI. Temsirolimus dosage was increased from 0 to 20.0 μM. All experiments were performed in triplicate for each condition and repeated at least twice. *$p<0.05$ at ≥5.0 μM in Caki-1 cells, ** $p<0.05$ at ≥2.5 μM in Caki-2

cells, two-tailed Student's *t*-test (C1-ctrl, native Caki-1 cell; C1-CCI, Caki-1 cell after CCI; C2-ctrl, native Caki-2 cell; C2-CCI, Caki-2 cell after CCI.)

2. Mitochondrial biogenesis enhanced after CCI

To explore the effect of CCI on RCC cells, we assessed changes of the mitochondria before and after CCI by observing the expression of mitochondria-specific protein voltage-dependent anion channel (VDAC). We observed that VDAC expression increased after CCI in both Caki-1 and Caki-2 cells. (Fig2A) When viewed by immunofluorescence staining, VDAC was prominently enhanced in the cellular cytoplasm of Caki-1 cells. The mean intensity of VDAC in Caki-1 cells was significantly higher after CCI, whereas that in Caki-2 cells showed no significant change after CCI. (Fig2B)

The functional status of mitochondria was assessed using a JC-1 mitochondrial membrane potential assay. In functional mitochondria with high mitochondrial membrane potential, JC-1 forms complexes known as J-aggregates with intense red fluorescence; whereas, in unhealthy collapsed mitochondria with low mitochondrial membrane potential, JC-1 remains in the monomeric form, which exhibits green fluorescence. The red-to-green ratio was used as an indicator of mitochondria function. Both Caki-1 and Caki-2 cells showed high proportion of J-aggregates. However, Caki-2 cells showed a significant decrease in J-aggregate:J-monomer ratio after CCI, implying decreased mitochondrial health. The J-aggregate:J-monomer ratio did not significantly change after CCI in Caki-1 cells. (Fig2C)

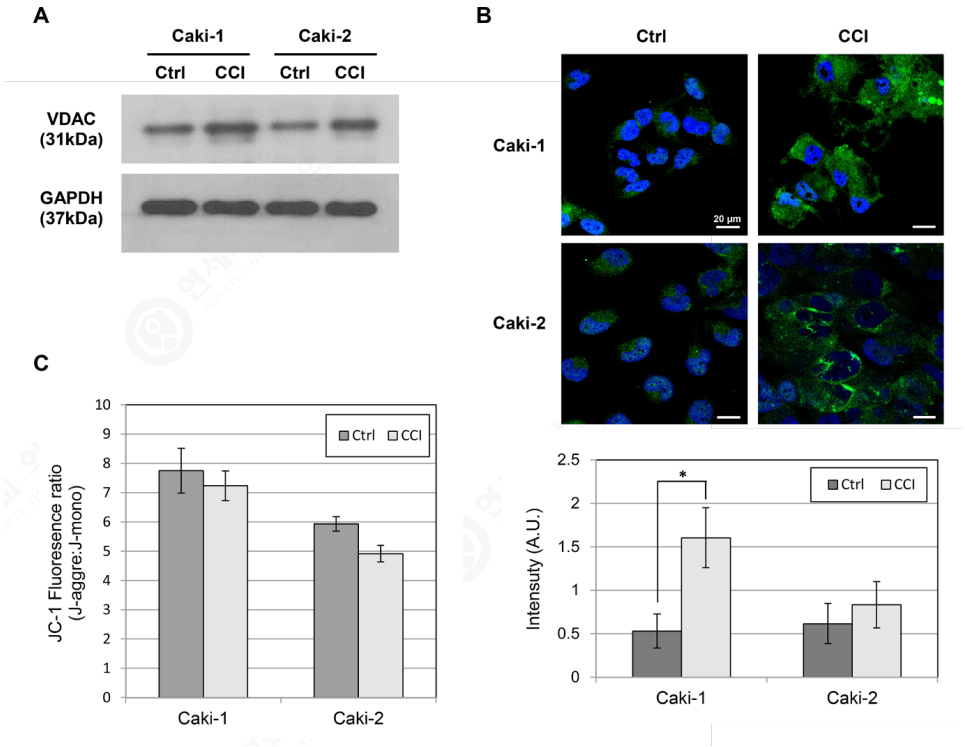


**Fig 2. Mitochondrial biogenesis enhanced after CCI.** A. Western blot of mitochondria-specific protein VDAC. B. Immunofluorescence staining and mean intensity of VDAC. *p<0.001, two-tailed Student's *t*-test. Green, VDAC; blue, DAPI. C. JC-1 mitochondrial membrane potential assay. *p=0.04, Student's *t*-test. (Ctrl=control, before clear cytoplasm induction; CCI=after clear cytoplasm induction.)

3. Adipogenesis genes differentially regulated after CCI

We studied the gene expression profile of 168 genes previously known to be related to adipogenesis and mitochondria (84 genes for each) by real-time PCR. Interestingly, 55 of the genes related to adipogenesis were distinctively upregulated or downregulated after CCI only in Caki-1 cells. (Fig3)

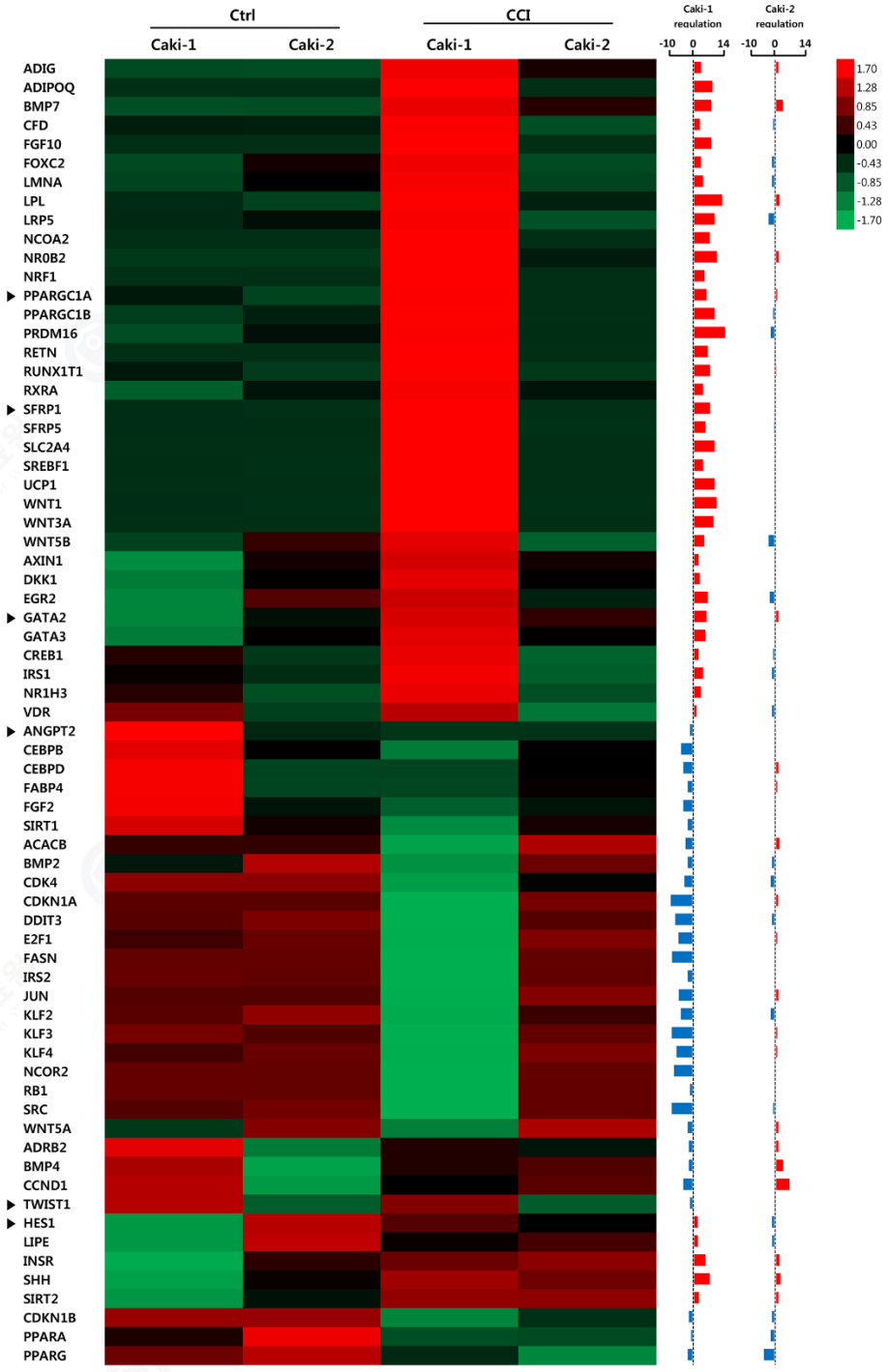

**Fig 3. Heat map of adipogenesis-related gene expression before and after CCI.** Only genes with more than two-fold change in expression are shown. The –ΔCT values are centralized and normalized across rows. The bar graph on the right represents the magnitude of fold change, described by base 2 logarithm of fold change of gene expression ($-\Delta\Delta CT = -[\Delta CT^{CCI} - \Delta CT^{ctrl}]$), before and after CCI in each cell line. (Ctrl=control, before clear cytoplasm induction; CCI=after clear cytoplasm induction.)

A literature search and analysis of these 55 genes revealed that many had already been reported to have an association with RCC. Notably, *DKK1*, *LRP5*, *SFRP1*, *SFRP5*, *WNT1*, *WNT3A*, and *WNT5B* are related to the Wnt/beta-catenin pathway, which is well-known to be associated with RCC. [9] The expression change of genes related to mitochondria was not as distinct as that of genes related to adipogenesis. (Fig4)

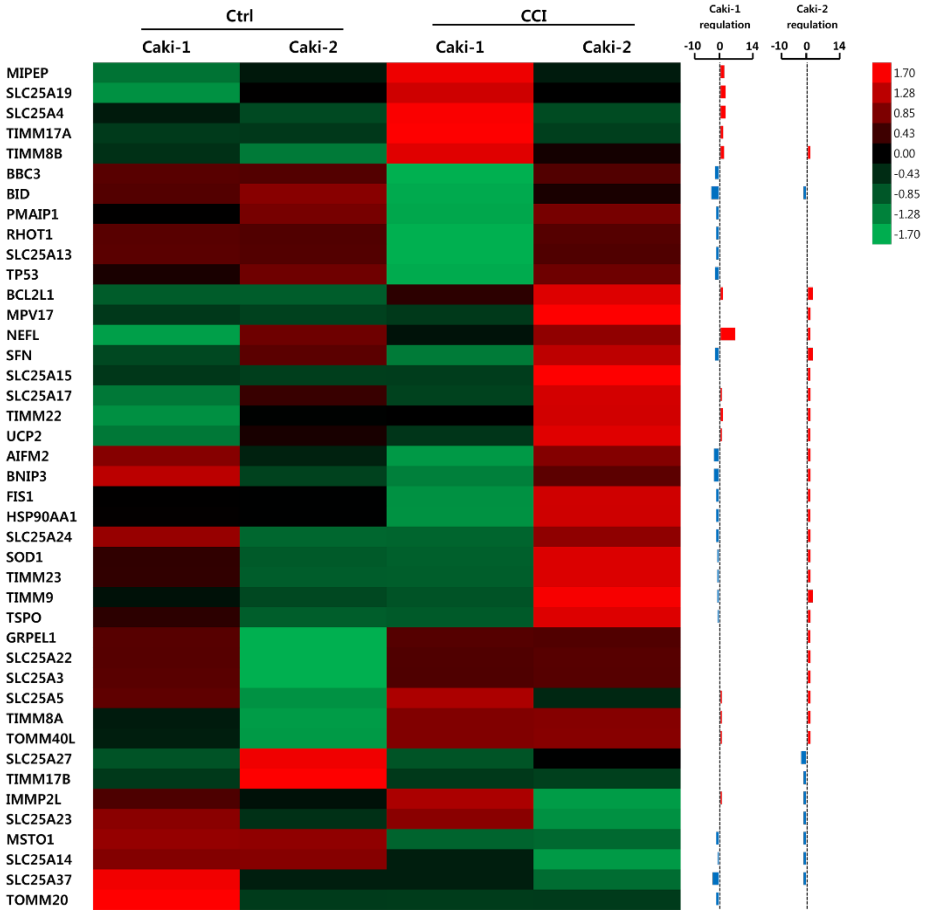


**Fig 4. Heat map of mitochondria-related gene expression before and after CCI.** Only genes with more than two-fold change in expression are shown. The –ΔCT values are centralized and normalized across rows. The bar graph on the right represents the magnitude of fold change, described by base 2 logarithm of fold change of gene expression ($-\Delta\Delta CT = -[\Delta CT^{CCI} - \Delta CT^{ctrl}]$), before and after CCI in each cell line. (Ctrl=control, before clear cytoplasm induction; CCI=after clear cytoplasm induction.)

4. PGC-1α expression is decreased in ccRCC patients

Genes with more than two-fold expression change after CCI were screened in our microarray dataset of ccRCC patients. The dataset consists of a

microarray analysis that compares mRNA expression in ccRCC tissue with that in normal kidney tissue from six patients. Seven genes screened from our dataset were found to have differential expression in ccRCC samples. (Fig5A, 5B) *SFRP1* encodes secreted frizzled related protein 1, which negatively regulates Wnt signaling and has been proposed to suppress tumor phenotype in RCC. [10] *GATA2* encodes GATA-binding protein 2, and its reduced expression has been reported to be associated with significantly shortened periods of recurrence-free survival and with adverse clinicopathology. [11] *TWIST1* encodes twist family bHLH transcription factor 1 and induces EMT in RCC cells in vitro and is positively associated with grade, pT stage, and metastasis in patients with RCC. [12] *PPARGC1A* encodes peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α); low levels of PGC-1α expression are associated with advanced stage tumors and metastatic disease in RCC patients. [7] *BNIP3*, which encodes BCL2 interacting protein 3, is a hypoxia-inducible HIF target gene known to promote mitophagy; its downregulation after CCI may be associated to the increased mitochondrial biogenesis observed in our study. [4]

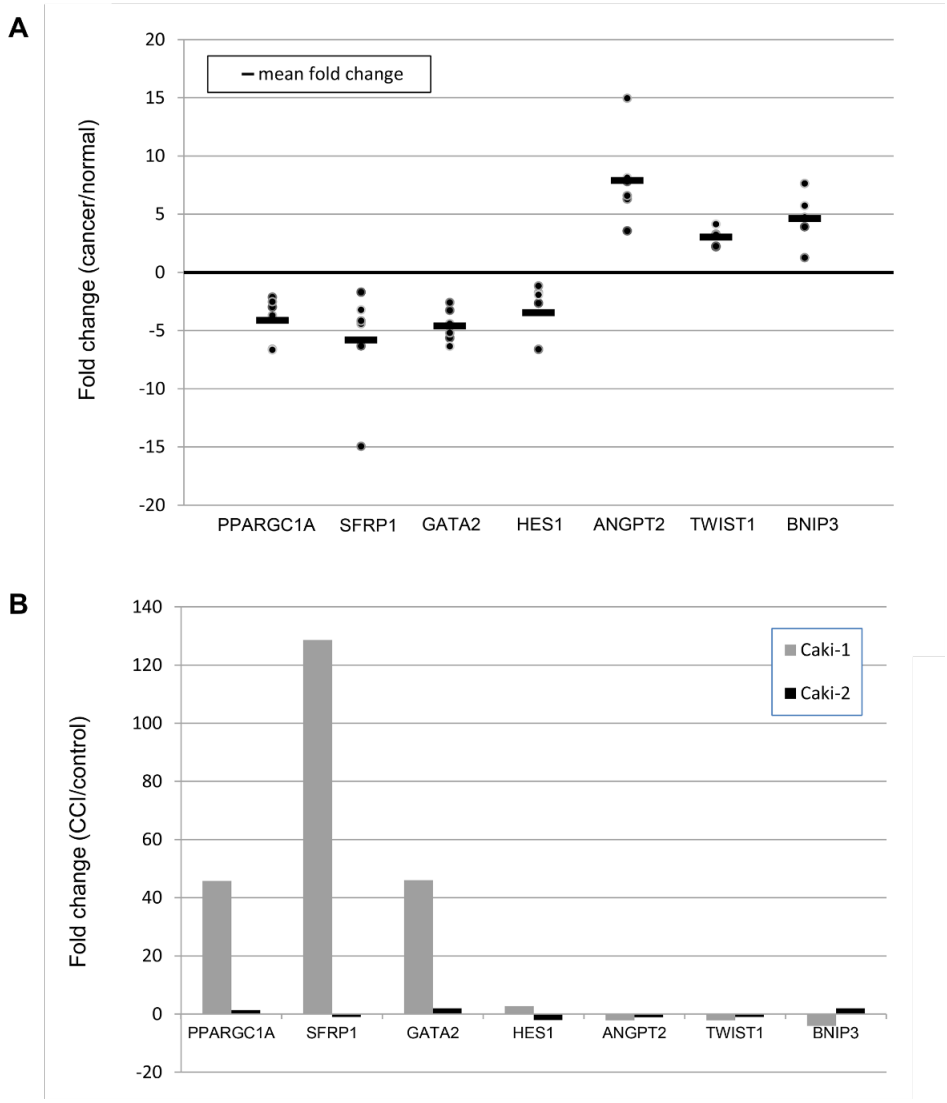


**Fig 5. Genes related to CCI were screened in our microarray dataset to compare mRNA expression of RCC tumor tissue and normal kidney tissue.** A. Fold change of cancer versus normal tissue gene expression in patients with kidney cancer. B. Fold change after CCI in Caki-1 and Caki-2 cells of the seven screened genes.

5. AMPK-PGC-1α pathway is activated by CCI

Among the genes with more than two-fold expression change after CCI, we further studied *PPARGC1A*, which encodes PGC-1α, a key regulator in energy metabolism and mitochondrial biogenesis. PGC-1α regulates mitochondrial biogenesis, oxidative metabolism, and gluconeogenesis, and has been implicated in multiple cancers. [13] RCC has been recently viewed as a disease of metabolism, with dysregulation in glycolysis and oxidative phosphorylation [14]. Further, the induction of PGC-1α in RCC cells has been reported to increase mitochondrial content, impair tumor growth, and enhance therapeutic response to chemotherapy and radiotherapy. [7]

Among many factors regulating PGC-1α, AMP-activated protein kinase (AMPK) takes a central role in energy metabolism. Interested in the role of AMPK as a regulator of mitochondrial biogenesis and lipid metabolism [15], we studied the expression of AMPK. In particular, we studied the expression of phosphorylated-AMPK (p-AMPK) before and after CCI. We found that expression of p-AMPK was prominently increased in Caki-1 cells compared to that of Caki-2 cells. (Fig6A) AMPK is also known to induce transcription of PGC-1α [16], which may explain why mRNA transcription of PGC-1α was more prominent in Caki-1 cells than in Caki-2 cells. Immunofluorescence staining of PGC-1α revealed that PGC-1α translation was also more prominent in Caki-1 cells after CCI than in Caki-2 cells. (Fig6B)

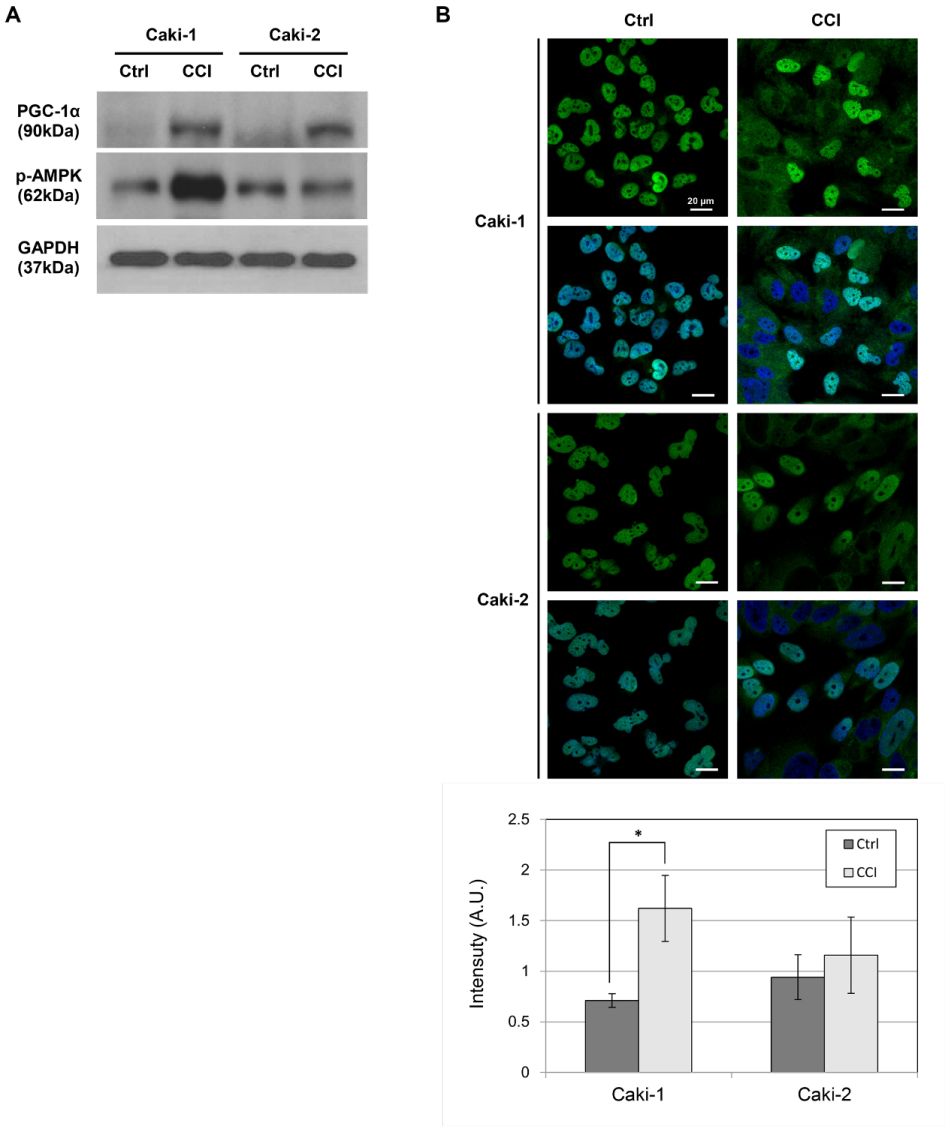


**Fig 6. AMPK-PGC-1α pathway is activated by CCI.** A. Western blot of PGC-1α and phosphorylated-AMPK. B. Immunofluorescence staining and mean intensity of PGC-1α. *p<0.001, two-tailed Student's *t*-test. Green, PGC-1α; blue, DAPI. (Ctrl=control, before clear cytoplasm induction; CCI=after clear cytoplasm induction.)

## IV. DISCUSSION

In this study, we investigated the significance of the clear cytoplasmic morphology of ccRCC. After CCI, Caki-1 cells featured a more prominent morphological change with distinct formation of lipid vacuoles compared to that in Caki-2 cells. The therapeutic response of Caki-1 cells to temsirolimus was enhanced after CCI, demonstrating that the effect of CCI not only changes morphology but also affects the tumor characteristics of RCC cell lines. We further investigated the changes induced by CCI. The expression of mitochondrial proteins was enhanced while maintaining adequate mitochondrial membrane potential, which indicated increased mitochondrial biogenesis. The expression of genes related to adipogenesis revealed a distinct pattern in Caki-1 cells after CCI. After reviewing the literature, we found that many of these genes are implicated in ccRCC, further indicating that the clear cytoplasm itself may affect RCC characteristics. These adipogenesis-related genes were cross-screened with our microarray dataset of ccRCC tissue samples, and *PPARGC1A* was proposed as a promising candidate for further studying.

Transcription of *PPARGC1A* was upregulated 45.8-fold after CCI in Caki-1 cells and decreased by 4.1-fold in tumor samples from patients with ccRCC compared to normal kidney samples. *PPARGC1A* encodes PGC-1α, which is well known to regulate mitochondrial function and lipogenesis. Expression levels of PGC-1α and p-AMPK were both increased after CCI in Caki-1 cells, suggesting activation of the AMPK-PGC-1α pathway. We thus conclude that the clear cytoplasmic morphology of ccRCC affects tumor characteristics such as drug response, and changes in tumor characteristics may be related to mitochondrial biogenesis and activation of the AMPK-PGC-1α pathway.

Clear cytoplasm, a distinct feature of ccRCC, is filled with lipids and glycogens. Given that dysregulated metabolism and lipogenesis are implicated in cancer cells, it is highly likely that clear cytoplasm is associated with the

pathophysiology of ccRCC. [1,14,17] However, the significance of clear cytoplasm in relation to oncogenesis has not been previously studied. In this study, we demonstrated that CCI changes oncologic characteristics of RCC cells in the absence of genetic mutation. This finding implies that ccRCC cells absent of clear cytoplasm, which are generally experimented on for in vitro studies, may not represent the actual ccRCC cells present in patients. As seen in our results, cancer cells that are resistant to therapeutic agents in in vitro studies can be responsive to therapy in patients. RCC cells with CCI can act as an in vitro model that is more representative of in situ ccRCC cells in patients. This effect of clear cytoplasm on tumor characteristics may also contribute to the different prognosis in ccRCC with variant histologic features, such as sarcomatoid features. [18,19] Further studies are needed to confirm that RCC cells with CCI not only resemble the morphology of ccRCC cells but also truly represent ccRCC cells in patients.

Although adipogenic induction prominently induced clear cytoplasm in Caki-1 cells, the same effect was not observed in Caki-2 cells. This result may be attributed to the fact that Caki-2 cells may derive from papillary RCC cell lines. Although this cell line was primarily defined as a ccRCC cell line, Caki-2 cells feature genetic characteristics of papillary RCC, including chromosome 8 aberration or high levels of *MET* and *LRRK2*. Furthermore, implantation of Caki-2 cells into nude mice results in cystic papillary tumors. [3] These findings support the assessment that the Caki-2 cell line is a papillary RCC cell line rather than a ccRCC cell line. Correspondingly, our finding that clear cytoplasm was not prominently induced in Caki-2 cells may be due to the papillary features of Caki-2 cells, which further supports such perspective. Further investigation on the ability of cells to develop clear cytoplasm may also allow this feature to be employed to validate whether a cell line is of ccRCC origin.

Caki-1 cells clearly displayed a distinct regulation of gene expression compared to that by Caki-2 cells, further underlining the difference between

both cell lines. Many of the genes shown to be regulated by Caki-1 cells have been reported to be related to RCC, and we further screened the genes in our microarray dataset to evaluate clinical relevance. In our study, the expression of *PPARGC1A*, *SFRP1*, and *GATA2* was decreased in tumor tissue compared to that in normal kidney tissue; these genes were upregulated after CCI. Expression of *TWIST1* in Caki-1 cells was higher in tumor tissue than that in normal kidney tissue and downregulated after CCI. Interestingly, reduced expression of the former three genes is known to be associated with adverse clinical parameters and prognosis in patients with RCC, and the genes have been proposed as tumor suppressors. [7,10,11] Expression of the latter gene is positively associated with adverse parameters in patients with RCC. [12] In short, after CCI of Caki-1 cells, genes associated with less aggressive cancers were upregulated, and the gene associated with a more aggressive cancer was downregulated. These findings support the theory that CCI by adipogenic induction in Caki-1 cells may have resulted in a cancer cell with less aggressive phenotype than that of the original Caki-1 cell devoid of clear cytoplasm. This may be related to our observation on the enhanced response to temsirolimus of Caki-1 cells with CCI.

We further focused on *PPARGC1A* as it encodes PGC-1α, which is well known to regulate mitochondrial biogenesis and lipogenesis. Although PGC-1α has been reported to contribute to cancer survival and therapeutic resistance, in contrast to most cancer types, low expression of PGC-1α in ccRCC is associated with poor survival. [7,20] A recent study by LaGory et al. demonstrated the association of PGC-1α expression and mitochondrial activity in ccRCC cells. The authors reported that in *VHL* wild-type cells, PGC-1α suppression decreased mitochondrial biogenesis and function, whereas rescued expression of PGC-1α in *VHL*-deficient ccRCC cell lines restored mitochondrial function. Furthermore, PGC–1α-induced mitochondrial respiration was associated with induction of oxidative stress and cells sensitized to cytotoxic therapies. [7] These

findings correlate with our results, as increased expression of PGC-1α is highly probable to be related to increased mitochondrial biogenesis. [21] However, because the therapeutic mechanism of temsirolimus differs from that of cytotoxic therapies, additional studies to elucidate this phenomenon are required.

We attempted to identify the upstream regulator responsible for enhanced PGC-1α expression. Among the many regulators of PGC-1α, AMP-activated kinase (AMPK) is a well-known moderator of PGC-1α, which activates PGC-1α by phosphorylation. [20] AMPK is also critically linked to the phosphatidyl-inositol-3 kinase/AKT/mTOR signaling pathway, which is a target for the treatment of RCC. AMPK activation directly inhibits phosphorylation and subsequent activation of the mTORC1 complex. [22] Because the kinase activity of AMPK is induced by phosphorylation of AMPK, we studied the expression of phosphorylated-AMPK (p-AMPK) and PGC-1α. Expression of both proteins was increased after CCI in Caki-1 cells, indicating activation of the AMPK-PGC-1α pathway. Because temsirolimus targets tumor cells by inhibiting the mTOR pathway, the synergistic effect of AMPK and temsirolimus on the mTOR pathway may have led to the sensitization of Caki-1 cells with CCI to temsirolimus.

Increased mitochondrial biogenesis after CCI may also be associated with downregulation of *BNIP3* in Caki-1 cells. *BNIP3* is known to promote mitophagy; therefore, its downregulation may result in increased mitochondria. *BNIP3* is also known to be hypoxia inducible and a target of HIF-1α. This is consistent with the fact that *BNIP3* was upregulated in RCC tissue compared to normal kidney tissue. HIF-1α is one of the most well-known proteins known to be associated with RCC tumorigenesis, and its increase may be associated with upregulation of *BNIP3* in RCC tissue samples. However, *BNIP3* has been reported to have tumor suppressor/metastasis suppressor function in mammary tumors and to be downregulated in lung, colorectal, hematologic, liver, and

pancreatic cancers. [4] The importance of *BNIP3* in RCC has not been well studied.

PGC-1α and BNIP3, encoded by *PPARGC1A* and *BNIP3*, are both known to be associated with mitochondrial function, and their association with various cancers has also been well studied. PGC-1α expression and BNIP3 suppression increase mitochondrial biogenesis and promote tumorigenesis, which may suggest that mitochondrial biogenesis is tumor promoting. [4] However, our study results conflicted with those from cancers originating from other organs. Although the association of the two genes with mitochondrial biogenesis was consistent with results from other studies, *PPARGC1A* was downregulated and *BNIP3* was upregulated in RCC tumors. This finding implies that the mitochondria may have a different role in RCC than that in cancers of other organs, and thus, further exploration is warranted.

Our interpretation of our results is limited given that adipogenic induction likely activates multiple pathways in addition to the AMPK/PGC-1α pathway. Further studies, such as selective inhibition of PGC-1α, will help clarify the role of AMPK/PGC-1α in RCC. The relevance of the mTOR/HIF-1α pathway also requires further investigation because AMPK and PGC-1α are both known to regulate and be regulated by this pathway. This may further elucidate the enhanced response of ccRCC to the mTOR inhibitor, temsirolimus. Inherent limitations are also present in this study because it was conducted on established cell lines. Further studies conducted on patient-derived cells and in vivo models are needed to confirm that in vitro RCC cells with CCI not only resemble the morphology of ccRCC cells but also truly represent ccRCC cells in patients.

## V. CONCLUSION

We believe that our results are significant, as we have shown that clear cytoplasm of ccRCC is more than a morphologic change—it affects expression

of numerous genes related to RCC. This point is emphasized by the fact that the therapeutic response of ccRCC cells with clear cytoplasm differs from that of cells devoid of clear cytoplasm. We suggest that mitochondrial activity and the AMPK/PGC-1α pathway play a significant role in the different tumor characteristics and thus, further investigation is warranted.

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ABSTRACT(IN KOREAN)

# 투명 세포질이 신세포암에 대하여 갖는 형태학적 특징 이상의 의의: 치료 반응, AMPK/PGC-1α 경로, 그리고 미토콘드리아와의 관련성

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나준채

투명세포질은 가장 흔한 신장암의 아형인 투명세포신세포암의 특징적인 조직학적 형상이다. 투명세포질의 병태생리학적인 의의에 대한 연구가 거의 보고된 바 없어서 본 연구에서는 투명세포질의 역할에 대하여 조사하였다. Caki-1과 Caki-2 신세포암 세포주를 지방생성 유발 배지에서 배양하여 투명세포질을 발현시켰다. 신세포암 세포주에서 투명세포질이 형성된 후에 temsirolimus 약제에 대한 반응이 증가하였고, 미토콘드리아 발생이 증가하였고, 그리고 지방생성과 관련된 유전자들의 발현이 차등적이었다. 투명세포질이 형성된 다음에 발현이 유의하게 변화한 유전자들을 투명세포신세포암 조직의 마이크로어레이 데이터에서 조사하였다. *PPARGC1A*, *SFRP1*와 *GATA2*는 정상 신장조직에 비하여 종양 조직에서 감소되어

있었고 본 연구에서 투명세포질이 발현하였을 때 이들 유전자들은 발현이 증가하였다. *TWIST1*은 정상 신장조직에 비하여 종양 조직에서 발현이 높았고 본 연구에서 투명세포질이 발현한 후에는 해당 유전자의 발현이 감소하였다. 마이크로어레이 데이터에서 조사된 유전자들 중 *PPARGC1A* 유전자를 추가로 조사하였다. 이 유전자는 미토콘드리아와 지방생성의 중재자 역할을 하는 PGC-1α 단백질을 부호화하고 있기 때문이다. 투명세포질을 발현시켰을 때 AMPK/PGC-1α 경로가 활성화된다는 것을 확인하였다. 결론적으로 투명세포질은 신세포암 세포의 치료제에 대한 반응에 영향을 주며 미토콘드리아 활동과 AMPK/PGC-1α 경로에 영향을 준다.

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핵심되는 말: 신장암, 투명세포신세포암, 투명세포질, 지방형성, 미토콘드리아, peroxisome proliferator-activated receptor gamma coactivator 1-alpha