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AUTOPHAGY SUPPRESSION POTENTIATES THE SUNITINIB-DEPENDENT INHIBITION OF CELL GROWTH AND MIGRATION IN KIDNEY CARCINOMA CELLS

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Introduction: Renal cell carcinoma (RCC) is a heterogeneous group of cancers arising from renal tubular epithelial cells. The most common subtype of RCC is clear cell RCC (ccRCC) that accounts for 70-80% of cases. Thirty percent of patients with RCC show metastasis at diagnosis and about 30% of cases develop disease recurrence after surgical resection. Many RCC patients treated with first-line vascular endothelial growth factor (VEGF) inhibitors such as sunitinib and pazopanib develop disease progression and require second-line therapy (1). The resistance to pharmacological treatment could be due to activation of autophagy. In fact, treatment with chemotherapeutic agents may cause metabolic stress that might induce autophagy, limiting the antitumor efficacy of these drugs (2). Despite the fact that autophagy has been

widely implicated as a form of cell death, especially in early stages of tumorigenesis, its role in cancer remains controversial. Cancer cells can use autophagy to survive in hostile metabolic microenvironment conditions, using autophagic substrates to sustain tumor growth and expansion (2). Therefore, the inhibition of autophagy could be used as a tool for the treatment of kidney carcinoma. Moreover, the suppression of this process could also enhance the effectiveness of conventional therapy. **Materials and Methods:** This study was performed by using kidney carcinoma cells (KJ29 and Caki-2) and SW-40 transformed normal epithelial kidney cells (4/5), used as control (3). The inhibition of autophagy was conducted infecting normal and ccRCC cells with lentiviruses containing the empty pLKO vector, used as control and the 394 and 395 recombinant pLKO vectors expressing specific shRNAs able to silence the autophagic ATG7 gene. The efficiency of ATG7 gene silencing as well as the inhibition of autophagy were evaluated by Western blotting by using specific antibodies for ATG7 and LC3II proteins, respectively. Cell proliferation was investigated by CellTiter cell proliferation assay (3). Briefly, normal (4/5) and ccRCC (KJ29 and Caki-2) cells were infected with either pLKO or 395 lentivirus for 24 h in DMEM/F12 medium with 1% FBS. Next, cells were cultured for further 48 h in the presence of 10 μ M sunitinib or DMSO (vehicle) and processed following the manufacturer's protocol. Cell migration was evaluated in Caki-2 ccRCC cells, treated as described above. Before the treatment with sunitinib or DMSO, pLKO and 395 transduced cells were grown at confluence in 24-well plates and a groove between the cells was generated using a sterile tip. Cell migration (groove filling) was analyzed after 48 h of culture with a phase contrast microscope equipped with a CCD camera and processed through the ImageJ software. Apoptosis was analyzed by the Hoechst method (3). Apoptotic nuclei were visualized by a fluorescence microscope after Hoechst staining of Caki-2 ccRCC cells treated with pLKO and 395 lentiviruses alone or in combination with sunitinib. Images were acquired by a CCD camera and processed using the ImageJ software. Statistical analysis was performed by ANOVA test. **Results and Discussion:** Because some advanced tumors have elevated levels of autophagy, it has been proposed as a form of tumor maintenance or promotion. Consistently, we have already described that ccRCC cells exhibit higher levels of autophagy than normal kidney cells. Therefore, increased autophagy could correlate with the progression of kidney carcinoma and it could be considered as a new prognostic marker for renal cancer. However, we have observed increased levels of autophagy not only in metastatic ccRCC tissues but also in some non-metastatic kidney tumors (data not shown), therefore autophagy could not be sufficient to promote tumor progression in kidney cancer. To evaluate the involvement of autophagy in the growth and progression of renal tumors,

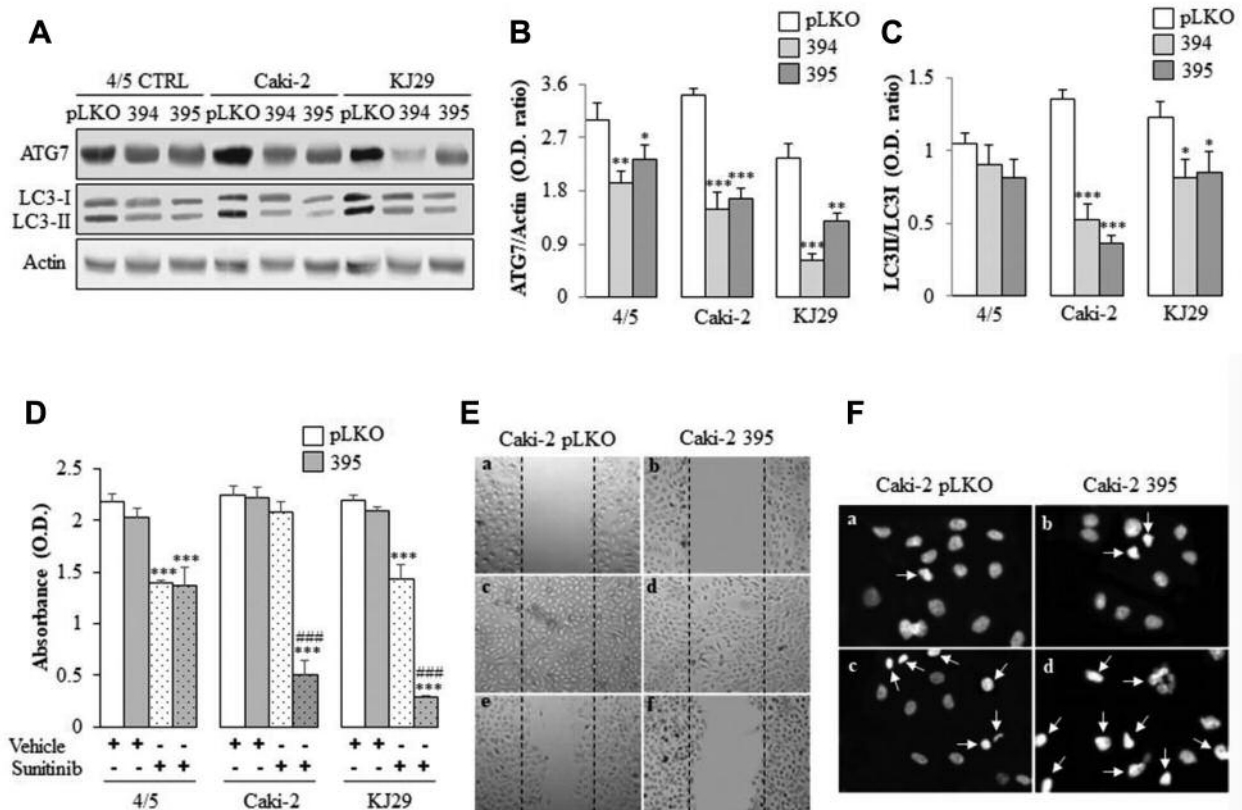


Figure 1. Inhibition of autophagy improves the efficacy of sunitinib causing the reduction of cell proliferation, the suppression of migration and the increase of apoptosis in ccRCC cells. Western blot analysis of normal (4/5) and ccRCC (Caki-2 and KJ29) cells shows the reduction of ATG7 (A and B) and LC3II (A and C) proteins in cells transduced with 394 and 395 (ATG7-silencing) lentiviruses compared to same cells transduced with pLKO (control) lentivirus. The statistical significance is * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (D) Normal and ccRCC cells were seeded at density of 5000 cells/well in a 96 well plate and infected with pLKO and 395 lentiviruses for 24 h. Next, cells were cultured for further 48 h in DMEM/F12 1% FBS in presence of vehicle (DMSO) or sunitinib (10 μ M) and analyzed by CellTiter method to evaluate cell growth. The treatment with sunitinib significantly reduced cell proliferation in all cell types except for Caki-2 cells (** $p < 0.001$). The combined treatment with 395 shRNA and sunitinib strongly reduces cell growth in both Caki-2 and KJ29 ccRCC cells but not in 4/5 control cells (### $p < 0.001$). (E) Cell migration was analyzed in Caki-2 cells infected with pLKO and 395 lentiviruses and cultured at 100% confluence in 24 well plates. Before the treatments, Caki-2 pLKO and 395 transduced cells were detached in order to generate a groove (panels A and B, respectively). Then, cells were cultured for further 48 h in DMEM/F12 1% FBS medium in presence of DMSO (panels C and D) and 10 μ M sunitinib (panels E and F). Images were acquired at $\times 10$ magnification. (F) Caki-2 cells were treated with pLKO and 395, as described above and cultured on glass coverslips in 6 well plates for 48 h in DMEM/F12 0.4% BSA in presence of DMSO (panels A and B) or with 10 μ M sunitinib (panels C and D). Images were acquired at 40x magnification. The white arrows indicate the apoptotic nuclei. Data were obtained from at least two independent experiments.

normal (4/5) and ccRCC (KJ29 and Caki-2) cells were infected with recombinant viruses containing specific shRNAs (394 and 395) able to inhibit autophagy by silencing of ATG7 gene. As shown in Figure 1A and B, the infection of 4/5 as well as KJ29 and Caki-2 cells with 394 and 395 viruses significantly reduces the levels of ATG7 protein compared with cells transduced with the control virus (pLKO). Consistently, the expression of the autophagic marker LC3II was decreased in 394 and 395 transduced cells, suggesting that the silencing of ATG7 gene causes the inhibition of autophagy in particular in Caki-2 and KJ29 ccRCC cells (Figure 1A and

C). We have observed that the inhibition of autophagy does not significantly change cell growth in normal and ccRCC cells (Figure 1D, gray bars). However, it has been postulated that the cancer cell could use autophagy to trap and degrade molecular drugs by lysosomal vesicles. Thus, the inhibition of autophagy should restore drug response. Consistently, the reduction of autophagy combined with sunitinib treatment, dramatically inhibits cell growth in both Caki-2 and KJ29 ccRCC cells compared with same cells untreated or treated with sunitinib alone (Figure 1D, gray dotted bars). Moreover, the combination between the suppression of autophagy and the

treatment with sunitinib in Caki-2 cells causes a greater inhibition of cell migration (Figure 1E, box f) compared to cells treated with sunitinib alone (Figure 1E, box e). In addition, the reduction of autophagy in Caki-2 cells improves the efficacy of sunitinib increasing the formation of apoptotic nuclei (Figure 1F, box d) compared with cells only treated with sunitinib (Figure 1F, box c). On the contrary, the only suppression of autophagy causes only small effects on cell migration and does not seem to be involved in the activation of apoptosis (Figure 1E box d and Figure 1F box b, respectively). These findings indicate that the inhibition of autophagy does not affect cell growth, migration and apoptosis in kidney cancer cells. However, the reduction of autophagy in combination with sunitinib treatment strongly reduces cell proliferation and migration as well as promotes apoptosis, removing the resistance of kidney cancer cell to the treatment with tyrosine kinase inhibitors. *Conclusion:* The data reported herein suggest that inhibition of autophagy potentiates the treatment with sunitinib, therefore, this biological process may induce drug resistance in kidney carcinoma cells. Moreover, suppression of autophagy combined with administration of anti-tyrosine kinase drugs could open new roads for the treatment of advanced kidney carcinoma.

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SBRT FOR LYMPH NODE METASTASES FROM PROSTATE CANCER: A MULTI-INSTITUTIONAL RETROSPECTIVE ANALYSIS

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Background: Metastases-directed treatment is an emerging strategy for oligometastatic/oligorecurrent/oligoprogressive lymph node metastases from prostate adenocarcinoma. Aim of the present study was to evaluate outcome of patients treated with stereotactic body radiation therapy (SBRT) on lymph node metastases. *Materials and Methods:* This is a multi-institutional retrospective analysis, including patients affected by lymph node metastases from prostate adenocarcinoma treated with SBRT. Patients with a maximum of 5 lymph node metastases were included. Concomitant treatment with systemic therapy was allowed. End-points of the analysis were local control (LC), out-of-field progression-free survival (OFPFS), overall progression-free survival (PFS) and overall survival (OS). *Results:* Eighty patients and 157 lymph node metastases, treated from 2009 to 2018 were evaluated. Median age was 70.2 years and median PSA before SBRT was 1.88 ng/ml. Median diameter of treated lesion was 37 mm (range 7-40 mm). Dose delivered ranged from 25 to 48 Gy in 5 to 12 Gy per fractions (median BED3Gy 116.67, range=66.67-240). Androgen-deprivation therapy was administered concomitantly in 72 lesions. With a median follow-up of 16 months, LC rates at 1 and 3 years were 93% and 86%. In-field progression of disease was observed in 11 (7%) lesions. One and 3-years OFPFS were 59% and 29% while PFS were 49% and 20%. Median values of OFPFS and PFS were 15 and 11 months, respectively. Rates of OS at 1- and 3-years were 100% and 95%. *Conclusion:* SBRT in the management of lymph node metastases from prostate cancer seems to be an effective approach with high rates of in-field control. Prospective trials are necessary to better select patients who can benefit the most from this ablative focal treatment.

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UPDATED RESULTS OF A PHASE II STUDY ON 5 FRACTIONS FFF SBRT FOR LOW AND INTERMEDIATE PROSTATE CANCER

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Background/Aim: SBRT has been shown to be a potential treatment option for localized prostate cancer (PC) in a