

Original Article

LC3B and ph-S6K are both expressed in epithelioid and classic renal angiomyolipoma: a rationale tissue-based evidence for combining use of autophagic and mTOR targeted drugs

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Abstract: Background: Targeted drugs to the autophagy processes are emerging in clinical trials. The aim of this work is to assess the magnitude of autophagic expression in renal angiomyolipoma. Methods: Fourteen cases of renal angiomyolipoma were recruited. Anti-LC3B-II and anti-phospho-S6K were detected by Western blot analysis. For immunohistochemical staining, sections were stained with the antibodies LC3B-II and cathepsin-K. LC3B-II was also analyzed by immunofluorescence. We have also carried out electron microscopy analysis on tumor cells. Results: 13 classic and 1 epithelioid renal angiomyolipoma were recruited. The Western-blot LC3B-II analysis shows increasing in protein expression in all cases, however quantitative protein expression ranged from 1 to 15 (mean 5). The autophagosome protein LC3B-I also significantly increased in all tumor extraction. The expression of LC3B-II protein was confirmed in tumoral samples by immunofluorescence. The lysosomal marker cathepsin-K was observed by immunohistochemistry on all tumours. The Western-blot ph-S6K analysis showed significant protein overexpression along all cases after evaluation of the quantitative S6K/Ponceaus ratio. In 6/14 (52%) the expression was high, with a quantitative increase of ≥ 3 fold induction in 4 angiomyolipoma compared to normal tissue. At electron microscopy, cancer cells evidenced round or oval electron-dense granules associated with membranes and granules with double membrane. Conclusion: Both autophagic LC3B-II and ph-S6K molecules are over-represented in both epithelioid and classic renal angiomyolipoma and a combined use of inhibitors to the autophagic and mTOR processes may be designed in clinical trials, when enrolling patients affected by tumours in tuberous sclerosis or angiomyolipoma at risk of bleeding.

Keywords: Renal angiomyolipoma, tuberous sclerosis complex, autophagy, ph-S6K, mTOR, LC3B

Introduction

Autophagy is a self-digesting mechanism that cells adopt following nutrient deprivation or stressful stimuli to deliver proteins and organelles to lysosomes, where they are degraded and recycled [1, 2]. Autophagy can act also as a protective mechanism during a stressful episode, however, if prolonged, it can lead to cell death, named 'autophagic cell death' [3, 4]. Recent studies have brought to attention the possible autophagy role in survival and growth

of cancer cells [5]. The cellular context may promote and/or inhibit autophagy in cancer cells. Notably, the well known mTOR protein complex is an autophagic process inhibitor and it is described constitutively active in the tumoral model of mTOR activation such as renal angiomyolipoma [6]. Renal angiomyolipomas occasionally occur sporadically and as part of tuberous sclerosis complex (TSC) and TSC is caused by mutations in either TSC1 or TSC2 suppressor genes [7, 8], resulting in increased mammalian target of rapamycin (mTOR) activity. The

research on autophagy as a mechanism for regulating cell death or survival has acquired huge interest in recent years, not only as a cell response to starvation but also as a mechanism involved in tumorigenesis, neurodegeneration and non-caspase-linked forms of cell death [2, 5, 9]. Even more importantly, drug inhibitors of the autophagy process have been developed and additional trials are under investigation in patients affected by malignant tumors. In the context of the kidney, the clinical relevance of using mTOR inhibitors for the treatment of angiomyolipomas is accepted, being tumors regressed somewhat during sirolimus therapy and some patients with lymphangioleiomyomatosis had improvement in spirometric measurements and gas trapping that persisted after treatment. Suppression of mTOR signaling constitute an ameliorative treatment in patients with the tuberous sclerosis complex or sporadic lymphangioleiomyomatosis.

Morphologically, autophagic cells show multiple cytoplasmic double-membrane vacuoles, called autophagosomes, which fuses with a lysosome to be degraded. Elongation of the autophagosomal membrane entails conjugation of MAP1-light chain 3B (LC3B) protein to phosphatidylethanolamine [10]. Few reports assess both activation of the LC3B and mTOR pathway in renal angiomyolipomas, by using different techniques on the same cohort of tumors. Assessment of biomarkers on the same tumors may promote use of combined targeted drugs or alternation of drugs which target to different pathways in patients affected by variants of renal angiomyolipoma.

The aim of this work is to verify the existence and magnitude of autophagic mechanisms in renal angiomyolipoma, which is one of the cancer model representing activation of the mTOR pathway in order to understand if administration of autophagic process inhibitors could have a rationale for a medical therapy in this lesion.

Materials and methods

Ethics statement

The University and Hospital Trust's Institutional Board approved all research involving human participants in accordance with the Helsinki Declaration of 1975. Informed consent was obtained in writing from living patients or rela-

tives for all tissues used in this study from the Transplant Kidney Center (Protocol PRIHTA 2014-00453).

Tissue samples

We selected from the Department of Diagnostics and Public Health, University and Hospital Trust of Verona, a series of renal angiomyolipomas, diagnosed by uropathologists (GM, MB, CG, AE) with morphology and panel of antibodies. Each tumor was accompanied by an adequate sample from the adjacent renal parenchyma, used as control.

Protein extraction and western blot analyses

After deparaffinization and rehydration of tissue sections, proteins were extracted using Qproteome FFPE Tissue Kit (Qiagen). Briefly, the area of interest was excised with a needle and transferred to a collection tube containing 100 µl of extraction buffer.

The sample was vortexed and incubated in 100°C water bath for 20 minutes and then in 80°C thermomixer for 2 h with shake at 750 rpm.

After heat-treatment, the sample was cooled to 4°C for 5 minutes and centrifuged (14000 g, 15 minutes, 4°C). The supernatant was transferred to a new collection tube and stored at -20°C.

Protein quantification was performed by using the Bio-Rad protein assay kit according to manufacturer's instructions. 25 µg of extracted lysates was resolved in 10% polyacrylamide SDS-PAGE gel in a BioRad Mini Protean tetra cell system at 150 V for 1 h.

Electrophoresed proteins were transferred into a nitrocellulose membrane at 250 mA for 90 minutes. The membranes were blocked in TBST (100 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20) plus 5% non-fat dry milk for 1 h at RT with constant shaking and probed overnight at 4°C with a rabbit polyclonal anti LC3B (1:1,000) (Cell Signaling, Danvers, MA, USA) antibodies, rabbit monoclonal anti-phospho S6K (1:1,000) (Cell Signaling, Danvers, MA, USA).

They were incubated O.N. at 4°C with the indicated antibodies, washed three times with TBST and incubated with the specific secondary anti-mouse or anti-rabbit peroxidase-conju-

Table 1. Clinico-pathological features of renal angiomyolipomas

Cases	Age	Sex	Localization	Synchronous localization	Diameter (cm)	Type
1	42	M	Polar localization	no	7.5	AML Classic
2	42	F	Mesorenal region	yes	1.4	AML Classic
3	42	F	Mesorenal region	yes	4.5	AML epithelioid
4	60	M	Polar localization	no	2.5	AML classic
5	52	F	Mesorenal region	yes	7	AML classic
6	61	F	Mesorenal region	no	6.5	AML classic
7	66	F	Polar localization	no	1.4	AML classic
8	60	F	Polar localization	no	7	AML classic
9	49	F	Mesorenal region	yes	4.2	AML classic
10	62	F	Polar localization	no	1,9	AML classic
11	76	F	Mesorenal region	no	1.3	AML classic
12	59	F	Mesorenal region	no	6	AML classic
13	69	F	Mesorenal region	yes	5.8	AML classic
14	31	F	Mesorenal region	yes	10	AML classic

gated anti IgG antibody. After three washes with TBST, the immunoblots were visualized with ECL plus (Amersham/GE HealthcareEurope GmgH, Munchen, Germany).

Expression levels of each marker was quantified with ImageJ densitometric analysis.

Immunofluorescence analyses

Paraffin-embedded tissue block were cut into 2-3 μ m sections and mounted on adhesion microscope glass slides. After the sections were dewaxed and rehydrated. Antigen retrieval was performed in prewarmed citrate buffer (pH 6 temp 95°C) for 30 minutes. The sections were cooled to room temperature and then incubated with a protein block serum free solution for 15 minutes at RT to block non-specific binding.

For immunofluorescence staining, sections were incubated with primary anti LC3B (1:100)

(Abgent, San Diego, CA, USA) antibodies, at RT for 60 minutes. Slides were then incubated with the secondary FITC-conjugated antibody (1:100) (Donkey, anti-rabbit Jackson Immuno-research) at RT for 30 minutes. Nuclei were stained with Prolong Gold antifade reagent with DAPI (Invitrogen Molecular Probe). Slides were analysed by a Olympus BX61 microscope.

Immunohistochemical analysis

Paraffin-embedded tissue block were cut into 2-3 μ m sections and mounted on adhesion microscope glass slides.

For immunohistochemical staining, sections were stained in an autostainer Leica Bond System with the following antibodies: LC3B Polyclonal (1:100) (Abcam, Cambridge, UK), Cathepsin K (1:300) (Abcam, Cambridge, UK), CD68 (1:50) (Dako, Carpinteria, CA, USA), CD68 (1:50) (Dako, Carpinteria, CA, USA).

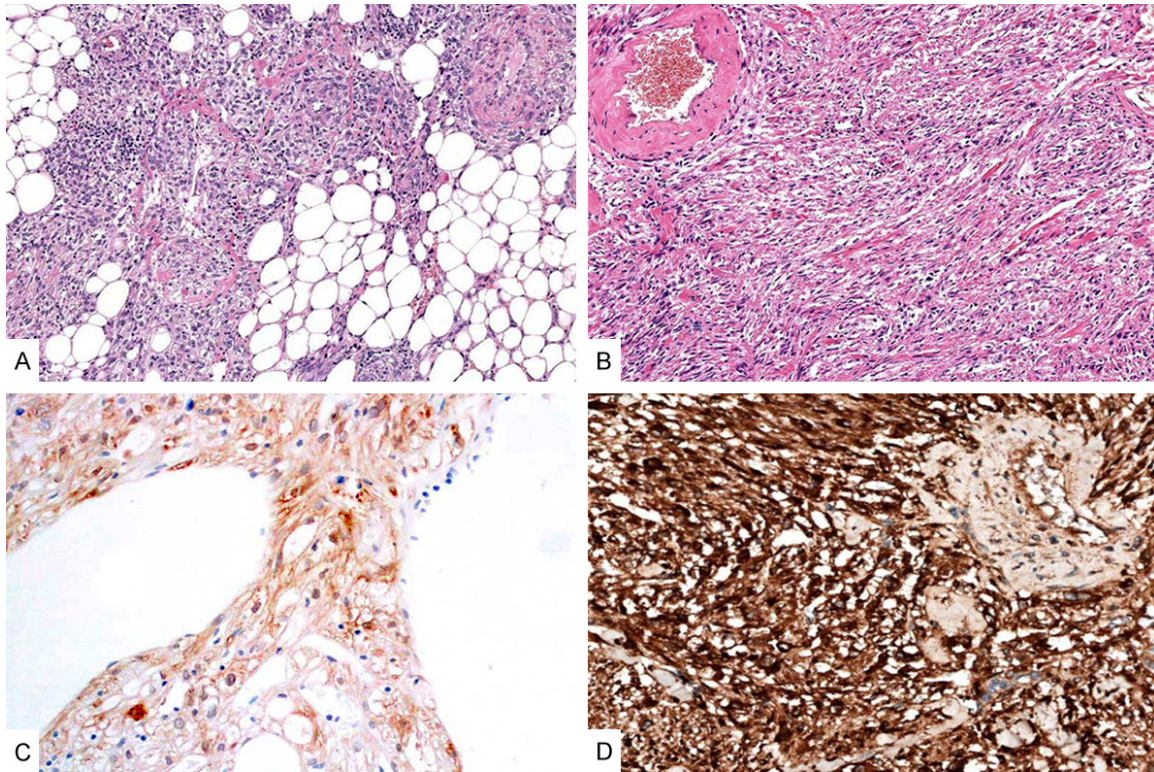


Figure 1. Histology and Immunohistochemical staining of Renal Angiomyolipomas: Hematoxylin and eosin stains showing a classic angiomyolipoma (A) a case with predominant epithelioid and muscle differentiation (epithelioid angiomyolipoma) (B). Lysosomal markers expression in renal angiomyolipoma samples. A section with expression of LC3B (C) and a case showing strong positivity for Cathepsin K (D).

Appropriate positive controls for each antibody were run concurrently and showed adequate immunostaining.

Electron microscopy

The tissue was fixed in formalin and then washed with phosphate buffer and fixed with glutaraldehyde at 2%; subsequently it was placed in 1% osmium tetroxide with ferric chloride potassium for 1 hour. Finally, the tissue was dehydrated with alcohol and associated in a mixture of Epon and Araldite.

Ultrathin sections were obtained in ultracut microtome (Reichert-Jung, Wien, Austria) by cutting with diamond, were stained with uranyl acetate of lead citrate, and finally valued at electron microscope Zeiss EM-10 (Oberkochen, Germany).

Results

Clinico-pathological findings

In **Table 1** the main clinico-pathological characteristics of 14 (Case no. 1-14) renal angiomyolipoma cases have been summarized.

13 classic angiomyolipoma and 1 epithelioid variant of renal angiomyolipoma (predominantly epithelioid and muscle differentiation) were recruited (**Figure 1A, 1B**).

The selected patients are ten females and two males aged between 31 and 76 years old.

Renal neoplasia had a diameter ranging from 1.4 cm to 10 cm. Polar localization in Case 1, 4, 7, 8 and 10 of the lesions were showed in the capsular region.

The lesions of case 2, 3, 5, 6, 9, 11, 12, 13 and 14 were located in the mesorenal region, as cortical/capsular localization.

Western blot finale results

The Western blot ph-S6K analysis shows significant protein overexpression along all cases after evaluation of the quantitative S6K/Ponceaus ratio.

In 6 out of 14 (42%) we observed high expression. In cases 1, 2, 4, 14 we have a quantitative increase of more than 3 fold induction in angio-

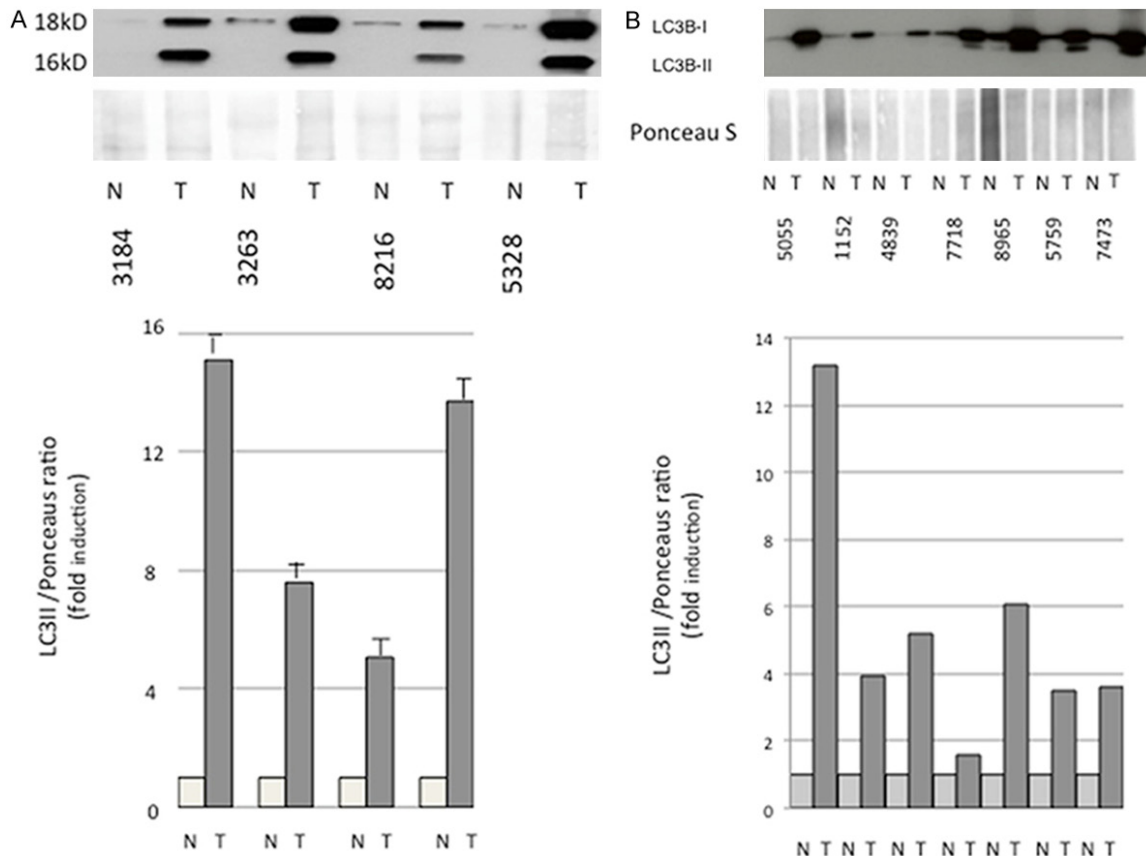


Figure 2. LC3B western blot in renal angiomyolipoma: A. Normal and renal angiomyolipoma protein extraction analyzed with western blot. The membranes were incubated with anti-LC3B antibody. Protein levels were normalized with Ponceau S staining. B. Quantitative evaluation on LC3B expression levels. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units. The value of Ponceau S was used as a normalizing factor. Values are the means of three independent experiments ($\pm$ SD). Statistical analysis: $P < 0.05$ LC3B versus CTRL.

myolipoma tissue compared to normal tissue, used as control. In case 13 a quantitative increase of more than 2 fold induction and a slight quantitative increase of fold induction in case 11 (**Figure 3**).

The Western blot LC3B-II (using anti LC3 protein antibody) analysis shows increasing in protein expression in all cases. Quantitative protein expression ranged from 1 to 15 (mean 5) (**Figure 2**).

The phosphoethanolaminated active form of the autophagosome protein LC3B-I, significantly increased in tumor tissues extraction (**Figure 2**).

Immunofluorescence finale results

The expression of LC3B-II protein was confirmed in tumoral samples with immunofluorescence analysis (**Figure 4**).

Immunohistochemical finale results

Lysosomal marker expression Cathepsin K were observed, by immunohistochemical analysis, in all cases in a form of strong expression (**Figure 1D**). Normal renal tissues were negative for any lysosomal markers.

Electron microscopy findings

Cancer cells were elongated, polygonal or rounded, with few intracytoplasmic material. In the cytoplasm it was observed glycogen, mitochondria, round or oval and electron-dense granules, associated with membranes. We also noted a number of granules with double membrane.

Discussion

We report that: 1) renal angiomyolipoma notably evidence an high expression of LC3B and

A ph ribosomal protein S6 kinase (ph S6K)

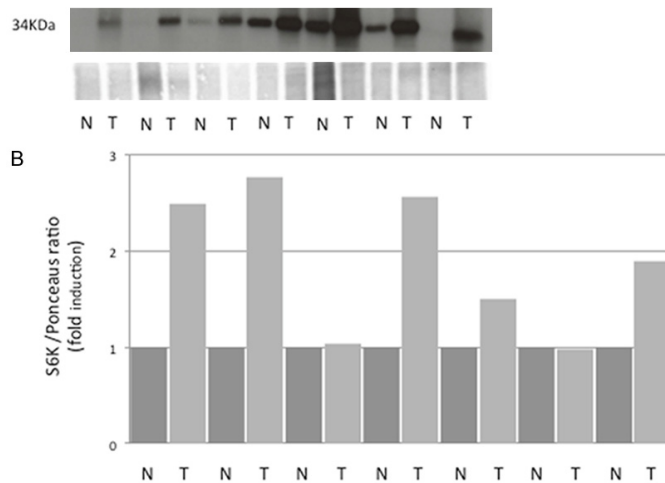


Figure 3. Phospho S6K western blot in renal angiomyolipoma: A. The normal and renal angiomyolipoma protein extraction analyzed with western blot. The membranes were incubated with anti-phospho S6K antibody. Protein levels were normalized with Ponceau S staining. B. Quantitative evaluation on Phospho S6K expression levels. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units. The value of Ponceau S was used as a normalizing factor. Values are the means of three independent experiments ($\pm$ SD). Statistical analysis: $P < 0.05$ Phospho S6K versus CTRL.

ph-S6K; 2) both autophagic and mTOR pathways are over-represented in both classic and epithelioid variant; 3) a combined use of mTOR and autophagic inhibitors may be designed in clinical trials for the enhancement of medical therapy when enrolling patients affected by tumors in tuberous sclerosis or angiomyolipoma at risk of bleeding.

We selected from our institute archives, 14 renal angiomyolipoma cases on which we made immunohistochemical, immunofluorescence and western blot studies to search protein involved in autophagy mechanisms (like LC3B-II) and factors able to adjust mTOR functional expression (like ph-S6K).

Angiomyolipoma, although is a benign tumor, can present risks. In fact, tumor size greater than 4 cm is associated with an increased bleeding risk, that can lead to retroperitoneal bleeding. Moreover, the presence of multiple lesions, characteristic event of the form associated with the tuberous sclerosis syndrome, may lead to progressive renal failure. There are, in the renal angiomyolipoma tumor group, rare forms with high malignant potential, such as the aggressive form of the epithelioid angiomyolipoma.

The ribosomal protein S6K (S6 kinase) represents an extensively studied effector of the TORC1 complex; therefore the presence of the phosphorylated form is used in order to verify the activation of mTOR, whose directed antibody is often not practical [11]. Moreover, western blot analyses showed increase levels of ph-S6K along all samples (Figure 3), thus mTOR pathway is activated in renal angiomyolipoma.

Notably, the mTOR protein complex is an autophagic process inhibitor and it has been reported constitutively active in renal angiomyolipoma tumor cells [12]. The autophagic process encounters different molecules along the pathway. In our study we shed light to a robust marker of autophagy such as the LC3B biomarker in renal angiomyolipoma and evidenced the activation in both the epithelioid and classic variants.

The induction of autophagy was further documented using anti LC3B-II protein antibody (Figure 2). The expression of LC3B-II protein, the phosphoethanol-aminated active form of the autophagosome protein LC3B-I, significantly increased in tumor tissues extraction and showed differences among the quantitative analysis (Figure 2). In 6 out of 14 (52%) we observed high expression: we observed a quantitative increase of more than 3 fold induction in 4 angiomyolipoma tissues compared to normal tissue, used as control and in one case a quantitative increase of more than 2 fold induction and a slight quantitative increase of fold induction in an additional case.

Again, the expression of LC3B-II protein was confirmed in all tumoral samples with immunofluorescence and immunohistochemical analysis (Figures 1C, 4).

There are several molecules that can inhibit at various levels autophagy, including chloroquine, an antimalarial drug [12, 13]. It blocks the fusion between autophagosome and lysosome and shows significantly reducing tumor growth in mice models [14]. mTOR inhibitors drugs are known as antitumoral in renal angio-

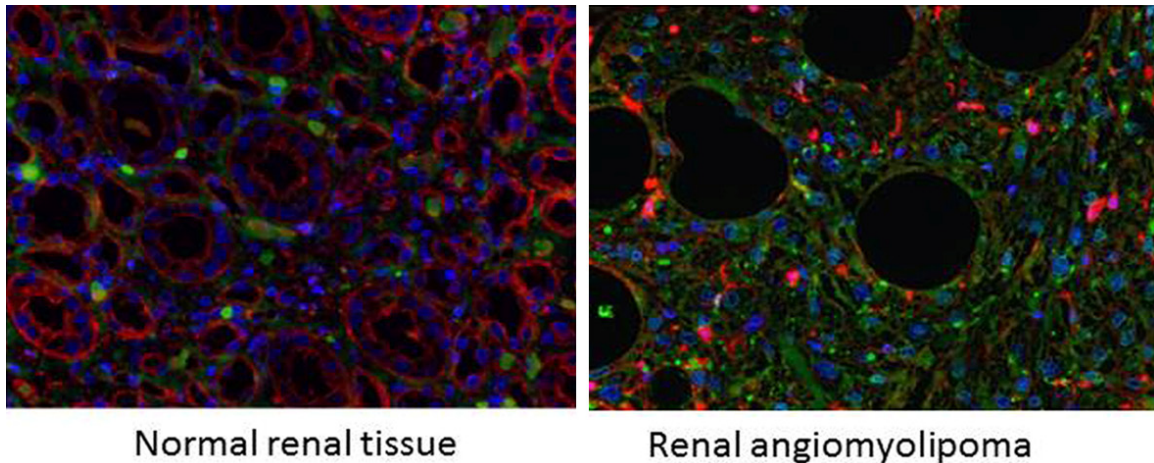


Figure 4. LC3B immunofluorescence in renal angiomyolipoma: Anti-human LC3B positive expression in normal and renal angiomyolipoma (immunofluorescence green).

myolipoma, such as rapamycin (Sirolimus); in the discontinuation drugs use is observed, however, a progression of the tumor [15].

Chloroquine and mTOR inhibitors drug combination, on experimental models, proved more effective than the single molecule use, this demonstrates a synergistic effect.

Microtubule-associated protein light chain 3B (LC3B) is a soluble protein with a molecular mass of ~17 kDa that is spread ubiquitously in mammalian tissues and cultured cells.

During autophagy, a cytosolic form of LC3B (LC3B-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3B-II), which is included in autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases.

At the same time, LC3B-II in autolysosomal lumen is degraded. Thus, lysosomal turnover of the autophagosomal marker LC3B-II reflects starvation-induced autophagic activity, and detecting LC3B by immunohistochemistry, immunofluorescence has become a reliable method for monitoring autophagy.

We have also verified the lysosomal markers presence in tumor cells by using cathepsin-k and CD68 [16]. Finally, we carried out a study by electron microscopy to verify double membrane organelles presence into the neoplastic cells cytoplasm [17-21]. At electron microscopy,

cancer cells were elongated, polygonal or rounded, with few intracytoplasmic material. In the cytoplasm it was observed glycogen, mitochondria, round or oval and interestingly electron-dense granules, associated with membranes. We also noted a number of granules with double membrane. In 1991 was described the constant expression of a melanogenesis marker in renal angiomylipoma (HMB45-gp100) and particularly in the epithelioid form of angiomyolipoma [17]. These intracytoplasmatic granules previously observed in angiomyolipoma, also observed in our study, were described by Yu et al. [22] as possible renin granules, and considered forms of pre-melanosomes. These granules were shown also present in clear cell sugar tumor of the pancreas [23] and in lymphangioleiomyomatosis [24]. Following this clarification, the CD68 lysosomal marker, in the classical form and in the epithelioid angiomyolipoma [25, 26] was initially reconsidered to be due to the presence of pre-melanosomes. Recently, the cathepsin K findings, a lysosomal protein, in angiomyolipoma cells tumor [27], again observed also in the actual study, suggested the relationship between granules to forms of lysosomes.

The assessment of the LC3B-II form or the relationship between LC3B-II and LC3B-I content is currently considered as a simple, quick procedure to verify the presence of cell autophagy [28] and its use is advised in recently published guidelines for the interpretation of assays monitoring autophagy [2].

In conclusion, in the actual study, we observed LC3B presence by immunofluorescence and western blot analysis. We verified the constant presence of LC3B-II in renal angiomyolipoma tumor cells. The increase of LC3B in this case may be explained by the activation of mTOR pathway and autophagy is not mutually exclusive. Both pathways may be at least in part activated in the tumor processes. Therefore, the autophagy process in renal angiomyolipoma constitutes a tissue-based rationale for using combination or alternative sequential use of autophagy inhibitors (such as cloroquine), and mTOR inhibitors in patients affected by pure epithelioid angiomyolipoma [29], lymphangi-oleiomyomatosis or patients affected by multiple angiomyolipomas in tuberous sclerosis syndrome or in angiomyolipoma (>4 cm) at risk of bleeding [26, 29].

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Disclosure of conflict of interest

None.

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