

Original Investigation

The mammalian target of rapamycin protein expression in human granulosa cell tumors

Güralp et al. mTOR protein expression in granulosa cell tumors

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Abstract

Objective: To investigate the role of Mammalian Target of Rapamycin (mTOR) in human granulosa cell ovarian tumors and the therapeutic effect of rapamycin in COV434 mitotic granulosa cell lines.

Methods: A retrospective evaluation of the medical records and pathological sections of patients with granulosa cell ovarian carcinoma was performed. mTOR and p-mTOR expression was immunohistochemically investigated. COV434 cell culture were treated with 0.5, 1, 2 and 5 μ M rapamycin. Real-time growth curve analysis via xCELLigence system and apoptotic cell analysis via YO-PRO™-1 Iodide were performed to assess the therapeutic effect of rapamycin on cancer cells.

Results: A total of twenty patients were evaluated. mTOR staining was detected in eighteen (90%) patients. Mild, moderate, intense, and very intense staining was observed in three (15%), eight (40%), six (30%) and one (5%) sample, respectively. Mean mTOR staining ratio was 59 \pm 41%. P-mTOR staining was observed in two (10%) patients. One (5%) patient had 5% staining, and one (5%) patient had 100% staining for p-mTOR. Both of the latter patients had “very intense” staining. Rapamycin caused a dose-dependent growth arrest and induced apoptosis in COV434 mitotic granulosa cells. The real-time growth curves of the cells treated with these drugs were distinguished by a marked reduced slope after exposure for several hours, indicating a rapid onset of apoptosis. Live/dead cell analysis with YO-PRO-1 staining showed that rapamycin induced apoptosis in 24% of the cells when used at 1 μ M concentration, whereas the rate increased to 61% and 72% when the cells were treated with 2 μ M and 5 μ M rapamycin, respectively.

Conclusion: mTOR expression is observed in various degrees in 90%, and p-mTOR expression is observed in only 10% of the patients with granulosa cell ovarian carcinoma. Rapamycin caused a dose-dependent growth arrest and apoptosis in COV434 mitotic granulosa cells.

Keywords: Granulosa cell ovarian tumor; mTOR; rapamycin; ovarian cancer

Introduction

Granulosa cell ovarian tumors constitute approximately 5% of all ovarian cancers. Contrary to the well-investigated epithelial ovarian tumors, there is very little known about the molecular and genetic changes in granulosa cell tumors. The studies are more or less focused on pathways playing a role in normal granulosa cell proliferation. The most important of these pathways is “the follicle stimulating hormone (FSH) pathway”. Recently, a somatic missense mutation was revealed in Forkhead box L2 (FOXL2) gene in 97% of the adult type granulosa cell tumors. Granulosa cell tumors are treated with surgery, adjuvant treatments (radiation or conventional chemotherapy), and hormone therapies (gonadotropin releasing hormone (GnRH) antagonists, tamoxifen and aromatase inhibitors). In metastatic or recurrent disease after primary surgical resection, adjuvant treatment is considered an option; however, the efficacy of the systemic chemotherapy remains controversial. Depending on our experiences on epithelial ovarian tumors, first line chemotherapy is platin-based chemotherapy. Agents like doxorubicin, cyclophosphamide, vinblastin, bleomycin, and etoposide were combined with cisplatin, and the response rates were detected to be between 60-83% (1). With a better

understanding of molecular and genetic features of granulosa cell ovarian tumors, the treatment options will certainly increase.

Mammalian Target of Rapamycin (mTOR) is a serine/threonine protein kinase and is a part of the phosphatidylinositol-3-kinase (PI3K)/AKT signal pathway. It plays a critical role in cellular development, metabolism, and cell cycle of cancer cells (2).

Disturbances of PI3K dependent signalling pathway may lead to a variety of tumors including ovarian, endometrial, and cervical cancer. In clinical studies, first line mTOR inhibitors were shown to have a promising clinical efficacy in ovarian and endometrial cancer (3). However, on a molecular level, the sensitivity or resistance rates of these agents are still unknown. At this point, we see that the studies on potential overactivity of mTOR pathway in granulosa cell ovarian tumors are scarce. In our study, we investigated the expression of mTOR and phospho-mTOR in granulosa cell ovarian tumor sections in order to better understand the molecular genetic features of granulosa cell ovarian tumors and determine whether mTOR inhibitors could be used in treatment. Moreover, we evaluated the possible therapeutic effects of Rapamycin in COV434 mitotic granulosa cell lines.

MATERIALS AND METHODS

The medical records and pathological sections of patients operated in xxxx (*intentionally blinded*) University, xxxx (*intentionally blinded*) School of Medicine, Gynecologic Oncology Clinic between 1999 and 2011 and diagnosed as granulosa cell ovarian tumor were evaluated retrospectively.

Our study was approved by xxxx (*intentionally blinded*) University, xxxx (*intentionally blinded*) School of Medicine, Medical Ethics Committee (date: 06 September 2011, no. 32476); and was supported by the xxxx (*intentionally blinded*) University, Scientific Research Fund of xxxx (*intentionally blinded*) University (Project number 17384).

In our search of medical records and pathology archives, we detected paraffin sections of 20 patients with granulosa cells ovarian tumor.

Pathological evaluation

Serial sections of 3 microns were cut from paraffin blocks by Thermo scientific® microtome (MI, USA). The sections were incubated and dried at 80°C for 20 minutes. Immunohistochemical staining was performed by streptavidin biotin method with fully automated Ventana Benchmark Ultra (Arizona, USA) immunohistochemistry staining instrument. Deparaffinization of the sections were completed by incubation in 72°C for 8 minutes followed by washing with Ez Prep (Ventana, USA). In the next step, sections were incubated with CC1 (Ventana, USA) at 95°C for 8 minutes for the antigen retrieval process. Sections were incubated with primary antibody mTOR (7C10) Rabbit mAb (1:50, Cell Signalling Technology) at 24°C for 48 minutes. Phospho-mTOR (Ser2448) (49F9) Rabbit mAb (IHC Specific) (Cell Signaling Technology, USA). Normal antibody Diluent (Scytek, USA) was used for antibody dilution. After treatment with primary antibodies, sections were treated with Blocker A and B (Ventana, USA) for 4 minutes each. Sections were incubated with biotinylated secondary antibodies (iView DAB Detection Kit, Ventana, USA) and streptavidin conjugated Horseradish peroxidase (iView DAB Detection Kit, Ventana, USA) for 8 minutes.

Sections were incubated with diaminobenzidine (DAB, iView DAB Detection Kit, Ventana, USA) and mordant application was performed by Copper (DAB, iView DAB Detection Kit, Ventana, USA). In all washing steps, Reaction Buffer (Ventana, USA) was used. Negative staining was performed by hematoxyline II (Ventana, USA) for 12 minutes. Sections were washed in tap water followed by alcohol baths. Xylene was used for making the sections translucent followed by covering with Consul-Mount (Thermo Scientific, UK) coverslip medium. Mammary ductal carcinoma in situ sections were used for positive mTOR. Normal mammary gland sections were used for positive P-mTOR.

Finally, sections were evaluated under light microscope (Olympus BX 50, OLYMPUS CORPORATION, Japan). M-TOR and P-mTOR expression were evaluated semiquantitatively; the “staining extent” was defined as the percentage of staining and the “staining intensity” was defined as “absent, weak, moderate, strong, and very strong”. The scoring scales were represented in Figure 1.A-F (percentage) and Figure 2 A-D (intensity).

Cell Culture

Human immortalized granulosa cell line (COV434) cells were maintained in Dulbecco's modified Eagle's medium:F12 (DMEM:F12) supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin Amphotericin B Solution (Gibco, 15240-062) at 37°C with 5% CO₂. The cells were routinely harvested by trypsinization with 0.25% trypsin-EDTA, counted using a hemocytometer and 0.4% trypan blue.

Real Time Growth Curve Analysis via xCELLigence System

xCELLigence System (ACEA Biosciences Inc. San Diego, CA, USA) was used according to manufacturer's instructions. Briefly, 100 µL of culture media was added to the each well, incubated at room temperature for 15 minutes and the background impedance was measured. Trypsinized COV434 cells were centrifuged, resuspended in complete media and seeded in 96-well E-Plate at the density of 10,000 cells per well in a final volume of 200 µL. The cells incubated at 37°C with 5% CO₂, continuously monitored on the RTCA system at 30-minute time intervals. When they reached the log growth phase, they were treated with 0.5, 1, 2 and 5 µM

concentrations of Rapamycine. The effects of Rapamycine on viability and proliferation of COV434 cells were monitored on the RTCA system for up to 200h. The results were expressed by normalized cell index (CI); which are derived from the ratio of CIs before and after the addition of the compounds. Recording and normalization of CI were performed using the RTCA Software 1.2.

Apoptotic Cell Analysis via YO-PRO™-1 Iodide

YO-PRO-1 is a carbocyanine nucleic acid stain used in identifying apoptotic cells. Apoptotic cells become permeant to YO-PRO-1 while live cells are not stained with YO-PRO-1. Culture media of the both control and Rapamycin treated cells were aspirated and replaced with YO-PRO-1 containing culture media (1 μ M). Hoechst 33342 was used as counterstaining. After 10 minutes of incubation at 37°C with 5% CO₂, they were observed under appropriate channels using an IF microscope (Olympus IX71, Japan).

Statistical evaluation

Parametric variables were expressed as mean \pm standard deviation; non-parametric variables were expressed as median, minimum and maximum; Student's T test and ANOVA were used for comparison of parametric variables; and chi-square test was used for comparison of nonparametric variables. Pearson correlation test was used for evaluation of possible correlations between parametric variables; Spearman correlation test was used for evaluation of possible correlations between non-parametric variables. Statistical Package for Social Sciences (SPSS 11.0, Chicago, IL, USA) was used for statistical evaluation. P<0.05 was accepted as significant.

RESULTS

A total of 20 patients with granulosa cell ovarian tumor were evaluated. Mean age was 46.05 \pm 11.5 (min: 26, max: 71). At the time of diagnosis, eleven (55%) patients were premenopausal and nine (45%) patients were post-menopausal. Eleven (55%) patients had stage 1a, five (25%) had stage 1c, one (5%) had stage 3b, and three (15%) had stage 3c disease. All patients had "adult type" granulosa cell tumor. Mean tumor size was 92 mm \pm 50 mm (min: 15 mm, max: 190 mm).

Necrosis was present in seven (35%) of the patients. Number of mitosis was 1-4 in seven patients, 4-8 in eight patients, and more than 8 in three patients. Nuclear atypia was absent in one (5%) patient. Eight (40%) had mild, five (25%) had moderate, and two (10%) had severe atypia.

mTOR staining was not seen in two (10%) patients. Three (15%) patients had mild, eight (40%) patients had moderate, six (30%) had strong, and one (5%) patient had very strong staining. Mean mTOR staining percentage was 59 \pm 41 (min: 0, max: 100). mTOR staining features were given in Figure 3 A-C.

There was no correlation between age and tumor size, mTOR staining percentage or intensity, p-mTOR staining percentage and staining. There was a positive correlation between mTOR staining percentage and staining intensity. (p<0.001, r=0.819) (Table 1)

P-mTOR staining was not observed in eighteen (90%) of the patients. One patient had 5% and the other one had 100% staining. Staining intensity was "very strong" in both patients. P-mTOR staining features were represented in Figure 4 A-B.

The patient with 5% p-mTOR staining was a 26-year-old woman with stage 3c disease, 5 cm tumor size, widespread tumor implants, 19 mitoses in 10 high power field (HPF), and high-grade atypia. The patient with 100% p-mTOR staining was a 42-year-old woman with stage 1a disease, 12 cm tumor size, 2/10 HPF mitoses, and low-grade atypia. The low number of p-mTOR positive cases rendered the statistical evaluation impossible. The growth curve characteristics of cells treated with different doses of Rapamycin were analyzed to observe cell proliferation/apoptosis rate. Compared to untreated control group, Rapamycin caused a dose-dependent growth arrest and triggered apoptosis in COV434 mitotic granulosa cells. The real-time growth curves of the cells treated with these drugs were distinguished by a marked descendent curve after exposure for several hours, indicating a rapid onset of apoptosis. (Figure 5A and B)

To further validate these findings obtained from xCELLigence system and confirm the apoptotic death after exposure to rapamycin, live/dead cell analysis with YO-PRO-1 staining was carried out.

Overall, rapamycin induced apoptosis in 24% of the cells when used at 1 μ M concentration, whereas the rate increased to 61% and 72% when the cells were treated with at 2 μ M and 5 μ M concentration, respectively. (Figure 6 A and B)

DISCUSSION

MTOR is abundant in cytoplasm and especially in the perinuclear area in normal granulosa cells (4). The kinase active serine 24-48 phosphorylated form of mTOR, in other words "the active form" p-mTOR, is generally increased during M phase. P-mTOR is observed near mitotic spindles and around contractile circle during cytokinesis. Inhibition of mTOR by rapamycin causes a dose-dependent decrease in granulosa cell proliferation and follicular development in vitro. However, in the presence of rapamycin, follicles do not undergo atresia in cell cultures. Yaba et al. (4) performed a study over rat ovaries and detected that mTOR expression was increased in cytoplasm compared to nucleus; they also showed that inhibition of mTOR in primary granulosa cell culture was associated with cell death in G2/M stages of cell cycle. Contrarily, granulosa cells survived in the presence of rapamycin, although tissue size was decreased. Therefore, rapamycin does not seem to stimulate follicle atresia directly, but it regulates follicular growth by acting as a check point.

Yu et al. (5) evaluated the mTOR expression in mouse ovaries, primary mouse granulosa cells, and spontaneously immortalized rat granulosa cell lines (SIGC). mTOR expression was best seen in M phase in primary mouse granulosa cells and SIGC groups. In mouse granulosa cells, p-mTOR was detected to be increased in G2/M phase. In a recent study by Rico et al. (6) mTOR inhibition was shown to slow tumor development in a transgenic mouse model. In our study, mTOR staining was not seen in two (10%) patients; whereas three (15%) patients had mild, eight (40%) patients had moderate, six (30%) had strong, and one (5%) patient had very strong staining. P-mTOR staining was not observed in eighteen (90%) of the patients. Detecting the stage of the cell cycle is possible in cell cultures but not in paraffinized sections. We cannot know for sure which stage of the cell cycle we observe when we stain the sections for mTOR and p-mTOR. Therefore, this may be regarded as an inevitable confounding factor.

Although the role of mTOR and p-mTOR in proliferation of granulosa cell and in vitro spontaneously immortalized granulosa cell line is well-established, no rigorously validated immunohistochemical study or targeted therapy on human granulosa cell tumors has been reported to date.

K-RAS oncogene is found in 48% of the borderline and serous ovarian tumors (7, 8). Stable transfection with H-RAS and other oncogenes may be used to immortalize the granulosa cells (9). However, since RAS mutations were never reported in ovarian granulosa cell tumors, the immortalized granulosa cell ovarian tumors may not represent the real human granulosa cell tumor proliferation mechanism. mTOR and p-mTOR are known to play a role in normal granulosa cell development and proliferation, but this pathway is not the only one maintaining protein expression. During cancer development, pathways other than mTOR could be activated.

Abnormal hyperstimulation of the pathways and oncogenic signalling are not the only pathologic mechanisms in cancer cell survival. Follicular growth and differentiation include complex mechanisms from the primordial stage until full establishment of corpus luteum; less than 0.1% of the follicles succeed. Female fertility depends on a delicate balance between survival signals for maturing follicle cells and death signals leading the other to undergo atresia. For this reason, a disturbance in apoptosis process may cause granulosa cell tumor development (1).

In cellular and molecular levels, ovarian cancer is known to be heterogenic. Altomare et al. (10) suggested that PI3K pathway, which also includes mTOR protein, was active in 70% of all ovarian cancer types and its up-regulation was the main interfering factor in drug resistance. However, in this pathway there are negative and positive feedback loops and alternative escape mechanisms interacting with the other pathways (11). For example, inhibition of mTORC1 by rapamycin leads to a short-term increase in mTORC2, which eventually increases hyperstimulation of AKT. Hyperstimulation of AKT opposes the suppressive effect of mTOR inhibition. Besides, inhibition of mTORC1 leads to a loss of effective feedback of p70 and IRS-1 on each other. In addition to the PI3K and AKT pathways that are activated by FSH, there is an alternative MEK/ERK pathway activated by tyrosine kinases. There are cross interactions between the abovementioned pathways. Although normal granulosa cell proliferation depends on FSH signalling, proliferation may be accomplished without FSH in carcinoma cells.

In order to evaluate the mTOR pathway in vitro, the growth curve characteristics of cells treated with different doses of Rapamycin were analyzed to observe mitosis/apoptosis rate. Compared to untreated control group, Rapamycin caused a dose-dependent growth arrest and apoptosis in COV434 mitotic granulosa cells. The real-time growth curves of the cells treated with these drugs were distinguished by a marked descendent curve after exposure for several hours, indicating a rapid onset of apoptosis.

To further validate these findings obtained from xCELLigence system and confirm the apoptotic death after exposure to rapamycin, live/dead cell analysis with YO-PRO-1 staining was carried out.

Overall, rapamycin induced apoptosis in 24% of the cells when used at 1 μ M concentration, whereas the rate increased to 61% and 72% when the cells were treated with at 2 μ M and 5 μ M concentration, respectively. These findings show that rapamycin may be a therapeutic option in vivo, however further studies are needed to assess this hypothesis.

Main limitation of our study is the limited number of patients. Studies with greater number of patients are needed to confirm the results of our study.

In conclusion, mTOR expression is observed in various degrees in 90%, and p-mTOR expression is observed in only 10% of the patients with granulosa cell ovarian carcinoma. Rapamycin caused a dose-dependent growth arrest and induced apoptosis in COV434 mitotic granulosa cells, which was confirmed with live/dead cell analysis with YO-PRO-1 staining. There is a strong need for studies on expression of mTOR and p-mTOR in human ovarian granulosa cell tumor cultures.

Funding

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Table 1. Correlations between age, stage, tumor size, presence of atypia and necrosis and mTOR staining percentage and intensity

	MTOR staining percentage		mTOR staining intensity	
	p	r	p	r
Age	0.750 ^a	-0.076 ^a	0.584 ^a	-.0130 ^a
Stage	0.128 ^a	0.352 ^a	0.109 ^b	0.370 ^b
Tumor size	0.296 ^a	-0.253 ^a	0.094 ^a	-0.395 ^a
Presence of atypia	0.868 ^a	0.043 ^a	0.443 ^b	0.199 ^b
Presence of necrosis	0.550 ^a	0.146 ^a	0.367 ^b	0.219 ^b
^a Pearson correlation test, p<0.05 is significant ^b Spearman correlation test, p<0.05 is significant r: correlation coefficient				

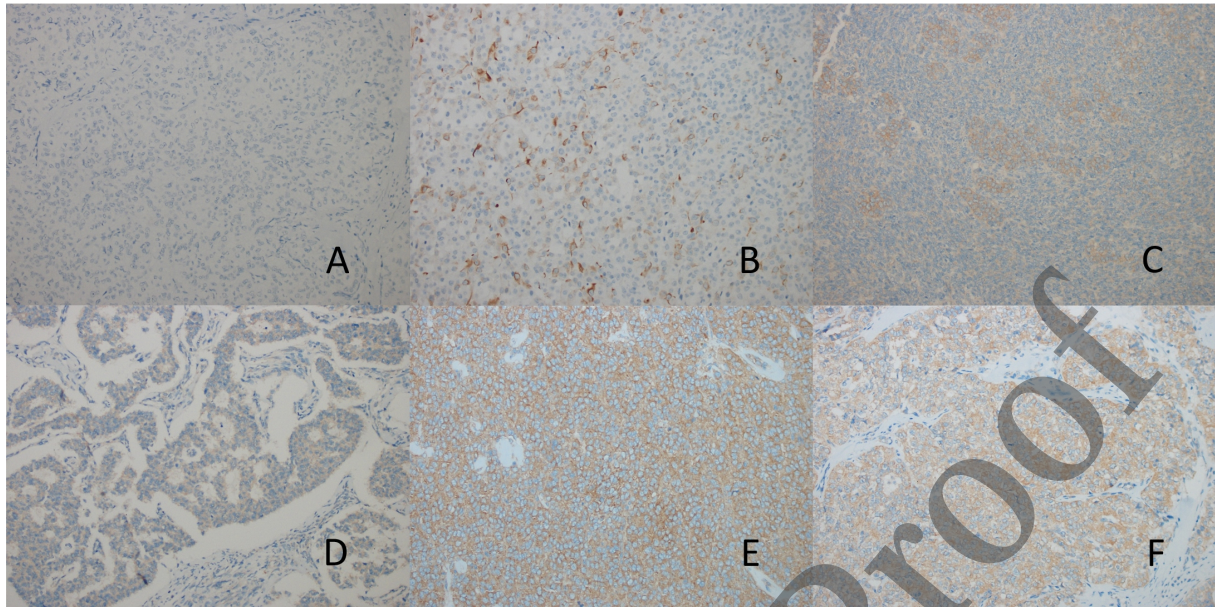


Figure 1 A-F: Scoring scales representation as percentage. mTOR and p-mTOR expression were evaluated semiquantitatively; the “staining extent” was defined as the percentage of staining. A. No staining (x200); B. 5% staining (x200); C. 30% staining (x200); D. 50% staining (x200); E. 90% staining (x200); F. 100% staining (x200).

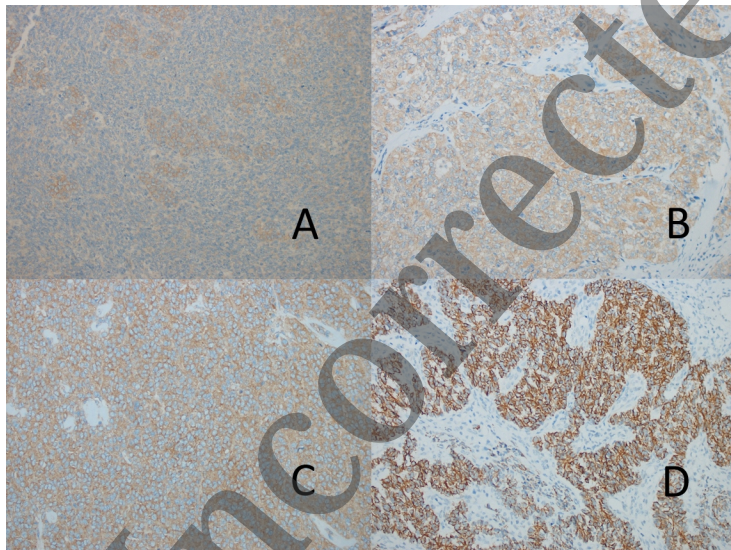


Figure 2 A-D: Scoring scales representation as intensity. The “staining intensity” was defined as “absent, weak, moderate, strong, and very strong”. A. (+) staining; B. (++) staining; C. (+++) staining; D. (++++ staining.

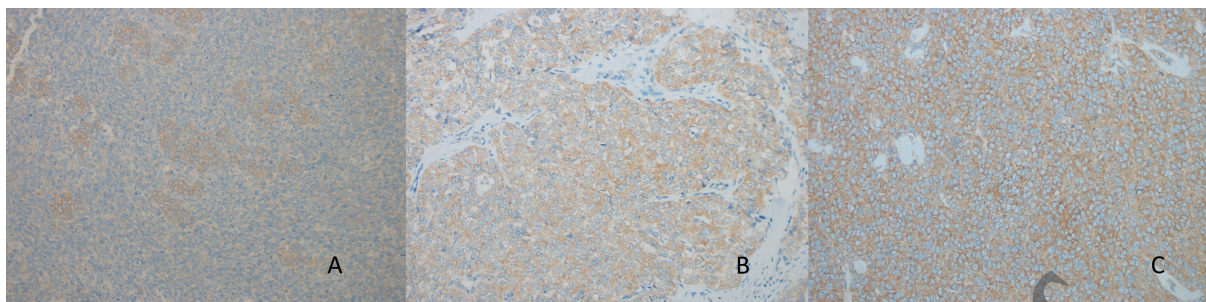


Figure 3 A-C: A. 30% (+) mTOR staining granulosa cell ovarian tumor (x200); B. 100% (++) mTOR staining granulosa cell ovarian tumor (x200); C. 90% (+++) mTOR staining granulosa cell ovarian tumor (x200).

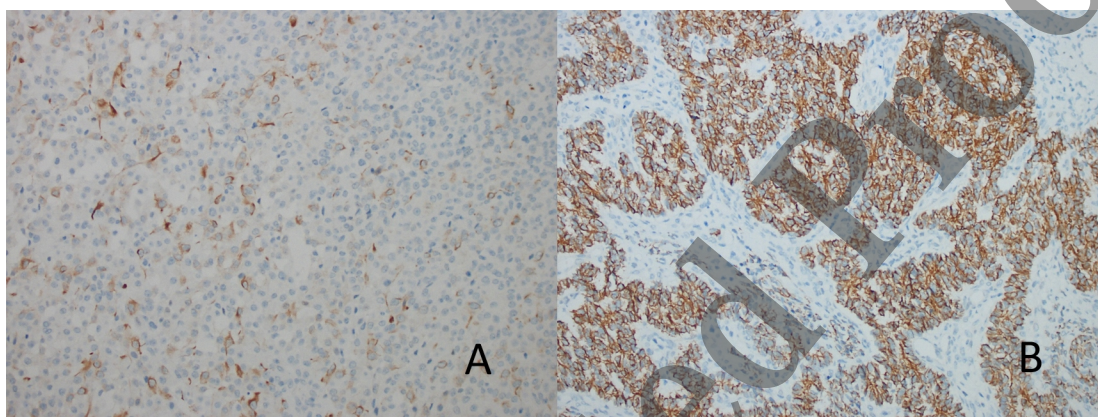


Figure 4 A-B: A. 5% (++++f) f-mTOR staining granulosa cell ovarian tumor (x200); B. 100% (++++f) mTOR staining granulosa cell ovarian tumor (x200).

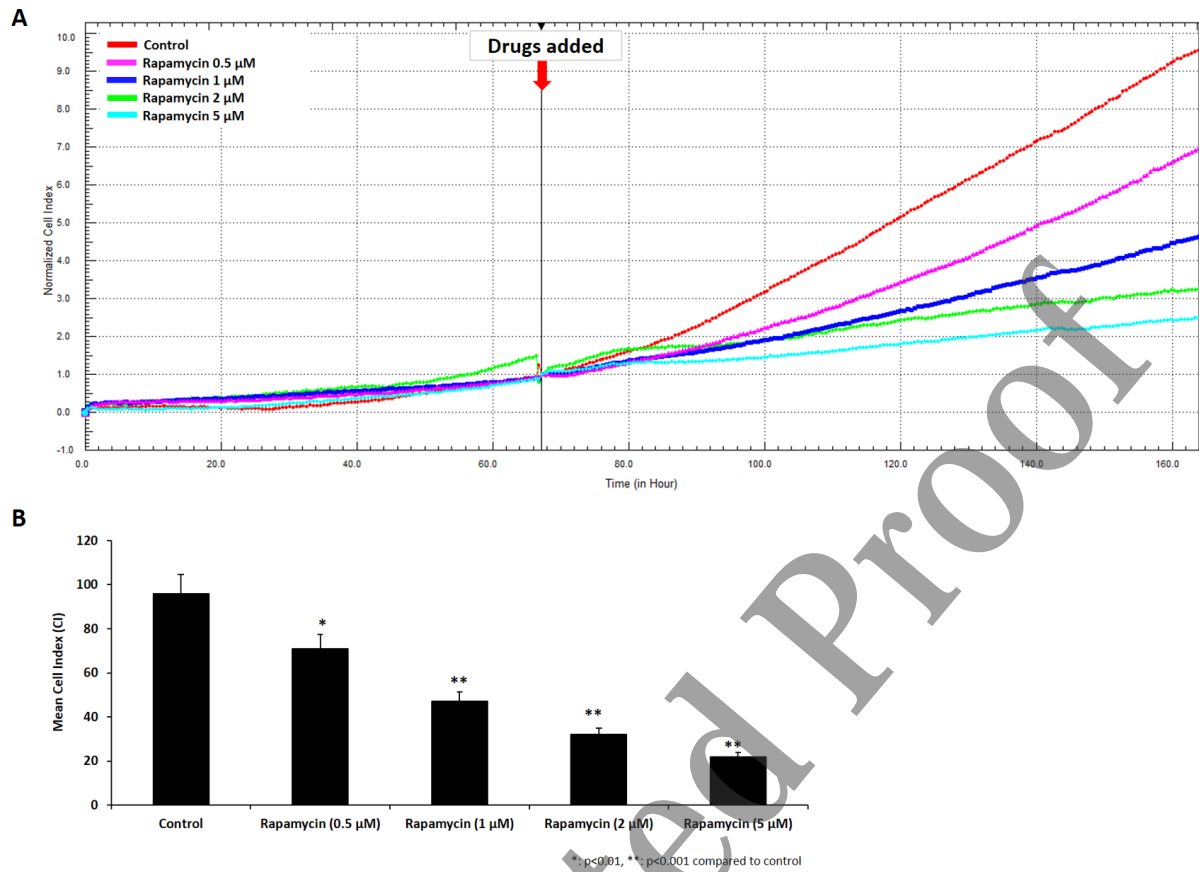


Figure 5 A-B: The real-time growth curves of the cells treated with these drugs were distinguished by a marked descendent curve after exposure for several hours, indicating a rapid decrease of the cell proliferation. A. The real-time growth curves of the cells treated with Rapamycin. B. Mean cell index according to the various Rapamycin doses.

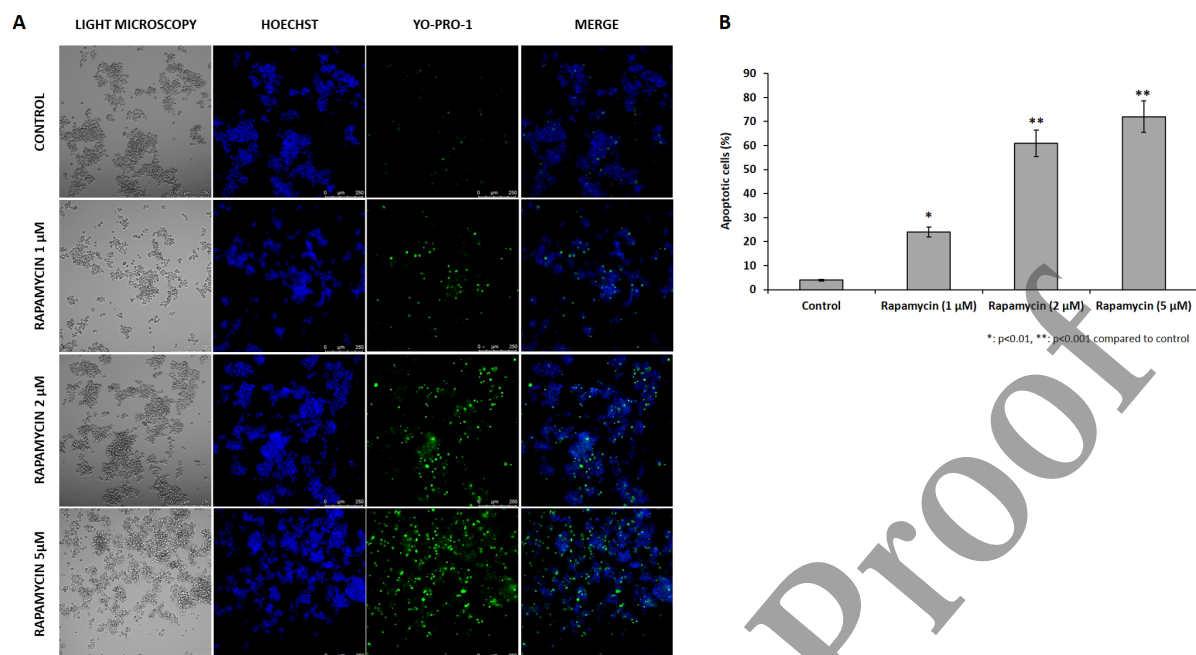


Figure 6 A-B: Live/dead cell analysis with YO-PRO-1 staining was carried out to confirm the apoptotic death after exposure to rapamycin. Overall, rapamycin induced apoptosis in 24% of the cells when used at 1 μ M concentration, whereas the rate increased to 61% and 72% when the cells were treated with at 2 μ M and 5 μ M concentration, respectively. **A.** Live/dead cell analysis with YO-PRO-1 staining **B.** Rate of apoptotic cells according to the various Rapamycin doses.