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Full Length Research Paper

Effect of chemotherapy with cisplatin and rapamycin on HeLa cells *in vitro*

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The aim of this study was to evaluate the effect of combination of rapamycin (RPM) with cisplatin on the proliferation of cervical cancer HeLa cells *in vitro*, as well as the expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF). HeLa cells were treated with RPM and cisplatin, respectively or through combining them for 72 h. There were four groups in this experiment, namely, RPM, cisplatin, RPM combined with cisplatin, and control group (culture medium only). The expression of mRNA and the protein of genes HIF-1α and VEGF were detected using reverse transcription-polymerase chain reaction (RT-PCR) and western blot respectively. A down-regulation of the mRNA and protein expression of HIF-1α and VEGF was observed in HeLa cells corresponding to the rapamycin group, cisplatin group, and RPM combined with cisplatin group compared with the control group ($p < 0.05$). The mRNA and protein expression of HIF-1α and VEGF were significantly down-regulated in the combined group. No significant difference was found between the RPM group and cisplatin group ($p < 0.05$). The mRNA and protein expression of both HIF-1α and VEGF were significantly down-regulated by the combination of RPM with cisplatin

Key words: HeLa cells, Rapamycin, cisplatin, hypoxia-inducible factor-1α, vascular endothelial growth factor.

INTRODUCTION

The prevalence of cervical cancer is the highest among malignancies, which involve the female genital tract. Although great progress has been achieved in its early detection, diagnosis, and treatment, the therapeutic outcomes of advanced cervical cancer remain unsatisfactory. Traditional surgical methods are mostly used for patients suffering in the early stage of the disease. Radiation therapy is adopted for the most intermediate and advanced patients, but it is helpless for recurring tumors. Neoadjuvant chemotherapy is mainly used for recurrent and advanced patients. Today, cisplatin is considered the first line of chemotherapy for cervical cancer. However, discontinuation of chemotherapy in advanced patients usually occurs due to obvious side effect and drug resistance (Hidalgo and Rowinsky, 2000). According to recent studies, rapamycin (RPM) and its derivatives can have an anti-tumor effect on numerous types of malignancies (Mondesire et al., 2004; Aleskog et al., 2008). Moreover, such a lethal effect only occurs in tumor cells rather than normal cells. Thus, nowadays, RPM has become a new type of safe, nephrotoxicity-free, and efficacious tumor inhibitor. Furthermore, the combination of RPM and cisplatin may become a new treatment protocol for cervical cancer. Such a combination can probably reduce the dose of cisplatin used in treatment, thereby alleviating cisplatin-induced toxic and side effects. The existence of hypoxic cells in cervical cancer tissue and the high expression of hypoxia-inducible factor-1α (HIF-1α) serve as important

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causes for various treatment failures (Bachtiary et al., 2003; Burri et al., 2003). HIF-1α can regulate the expression of different target genes, including coding vascular endothelial growth factor (VEGF), maintain the energy metabolism of cancer cells, affect neovascular formation, and promote the proliferation and metastasis of cervical cancer (Blagosklonny, 2001). Therefore, the antagonistic activity of HIF-1α may be a potential target for cancer therapy. Recent studies have shown that mammalian target of RPM (mTOR) inhibitor can block HIF-1α expression in the transcription and translation levels, resulting in an anti-tumor effect (Hudson et al., 2002; Jiang and Feng, 2006). In the current study, subtoxic doses of RPM and cisplatin were combined to evaluate the inhibitory effect of such treatment on HeLa cell line *in vitro* by determination of the expression levels of HIF-1α and VEGF. The mechanism of tumor vascularization inhibition via inhibiting the expression of HIF-1α and VEGF was further explored to provide a theoretical basis for new combination chemotherapy for cervical carcinoma.

MATERIALS AND METHODS

Cell cultures

HeLa human cervical cells were provided by the Department of Histology and Embryology of Dalian Medical University. They were cultivated in RPMI-1640 DMEM combination, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/L streptomycin, in 5% CO_2 at 37°C. The growth of cells was then recorded. When 70% to 80% cells reached adherent growth, the cells were digested with trypsin and then pre-cultivated. For further study, the cells in the logarithmic growth phase were used for further study.

Drug intervention

The cells were released by using trypsin treatment, and then counted a day before the drug intervention. When drugs were used, cells were transferred into the 96-well plates (concentration: 2×10^6/ml) to reach 70 to 80% density. After adherent growth, drugs of different concentrations were added, and then the cells incubated in 5% CO_2 at 37°C for 24 h.

There were four groups in this experiment, namely, control group (culture medium only), RPM group (20 nmol/ml), cisplatin group (0.5 mg/ml), RPM (20 nmol/ml), and combined cisplatin group (0.5 mg/ml).

Reverse transcription-polymerase chain reaction (RT-PCR)

Trizol method was used for extraction of total cell RNA for 48 h after drug intervention in all groups previously described. Samples in amounts of 0.25 μg RNA were treated with reverse transcriptase. HIF-1α and VEGF mRNAs were amplified by RT-PCR. As an internal control, β-actin mRNA was also similarly amplified. The HIF-1α up-stream primer sequence was 5′-AACAAAAACACAGCGAAGC-3′, the downstream primer sequence was 5′-ATAGTGAATGTGGCCTGTG-3′, and the product length was 124 bp. The VEGF upstream primer sequence was AGGGCAGAACATCACGAAG-3′, the downstream primer sequence was 5′-ACTCCAGGCCCTCGTCATTG-3′, and the product length was 182 bp, respectively. Analogically, the β-actin upstream primer sequence was 5′-TCCTTCTGCATCCTGTCGGCA-3′, the downstream primer sequence was 5′-CAAGAGATGCCACGGCTGCT-3′, and the product length was 275 bp. Primer synthesis was accomplished by TaKaRa Biotechnology Co., Ltd. PCR procedure was performed as follows: 35 cycles at 50°C for 30 min, 94°C for 2 min, 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. RT-PCR products were detected by 2% agarose electrophoresis and recorded using UVP Gel Imaging System. Data were analyzed with Labwork 4.6 image acquisition and the relative expression of two genes using HIF-1α/β-actin and VEGF/β-actin were shown by analysis software.

Western blot assay

Total protein was extracted from HeLa cells from each group, and its concentration was detected by using the bicinchoninic acid (BCA) protein assay kit. The protein levels were assayed by the standard curve. The mixture (80 μg total protein, 5× sample buffer and additional water to reach to 20 L) was boiled for 5 min. Proteins were separated after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), moved to the polyvinylidene fluoride (PVDF) membrane, and then immersed in the transferation fluid for 30 min. The transferation time was 120 min for HIF-1α, 45 min for VEGF, and 60 min for β-actin, respectively. Then, the PVDF membrane was placed in skimmed milk powder for 2 h at room temperature. It was subsequently placed in mouse anti-HIF-1α monoclonal antibody (1:100), mouse anti-human VEGF monoclonal antibody (1:200), and rabbit anti-β-actin antibody (1:2000) at 4°C overnight. The membrane was washed 3 times for 10 min with cold phosphate buffered saline with Tween-20 (PBST containing 1‰ Tween-20). Then, it was incubated with secondary antibody for 1 h at 37°C. The membrane was washed 3 times for 10 min with cold PBST (containing 1‰ Tween-20). The membrane was detected by electrochemiluminescence (ECL) and X-ray colour reaction, and then washed. The gray value was detected with the UVP Gel Imaging System.

RESULTS

HIF-1α and VEGF mRNA expressions

The results from RT-PCR indicated that mRNA expressions of HIF-1α and VEGF significantly decreased in RPM group, cisplatin group, and RPM combined with cisplatin group compared with the control group, and the difference was statistically significant ($P < 0.05$). The integral optical density (IOD) value of each band was recorded using UVP Gel Imaging System (Figures 1 and 2).

The data analysis indicated that mRNA expressions of HIF-1α and VEGF in the cervical cancer HeLa cells were significantly down-regulated in RPM group, cisplatin group, and RPM combined with cisplatin group compared with the control group, and the difference was statistically significant ($P < 0.05$). The mRNA expressions of HIF-1α and VEGF were significantly down-regulated in the combined group compared with RPM and cisplatin, and the difference was statistically significant ($P < 0.05$). There was no significant difference in the mRNA expressions of HIF-1α and VEGF between RPM and cisplatin

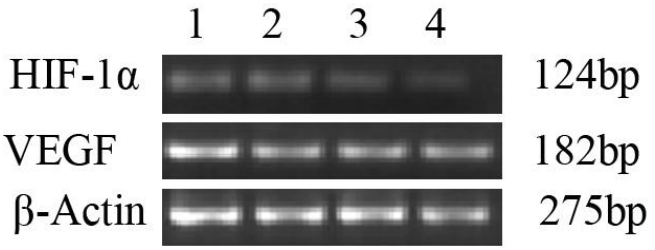

Figure 1. The amplification band of HIF-1α mRNA and VEGF mRNA. 1, control group; 2, RPM group; 3, cisplatin group; 4, combination group.

groups (P > 0.05) (Table 1).

HIF-1α and VEGF protein expression

As per the results of western blot assay, the protein bands were clear in each group, but they were weaker in the combination group. The IOD value of each band was recorded by using UVP Gel Imaging System (Figures 3 and 4).

The data analysis indicated that the levels of both HIF-1α and VEGF protein expressions in cervical cancer HeLa cells were significantly down-regulated in RPM group, cisplatin group, and RPM combined with cisplatin group in comparison to the control group ($p < 0.05$). Similarly, the protein expressions levels of HIF-1α and VEGF were significantly decreased in the combined group in comparison to the RPM group or cisplatin ($p < 0.05$). There was no significant difference in the levels of protein expressions of HIF-1α and VEGF between RPM and cisplatin groups ($p > 0.05$) (Table 2).

DISCUSSION

VEGF is believed to play an important role in tumor angiogenesis and in the increased incidence of tumor metastasis by releasing tumor cells into the vasculature, promoting vascular endothelial proliferation, increasing capillary permeability, and modifying the extracellular matrix (Minet et al., 2000). Many researchers have shown a close relationship between the high expression of VEGF and its receptor in gynecologic malignant tumor tissues closely related to tumor invasion and metastasis (Sivridis et al., 2002). The high expression of HIF-1α and VEGF in the cancer tissues of patients with epithelial ovarian cancer, endometrial cancer, and cervical is closely related with the tumor prognosis (Sivridis et al., 2002; Sonode et al., 2003; Wong et al., 2003). Angiogenesis in cancer masses has been established to be induced mainly by up-regulation of VEGF and HIF-1α expression, and hence, higher transcriptional activities (Carmeliet et al., 1996; Bos et al., 2001). Therefore, the inhibition of the expression of HIF-1α and VEGF may be a new tumor treatment strategy through the anti-angiogenesis mechanism.

As closely related with the tumor, the development has been characterized by Akt/mTOR signal pathway, mainly by cell cycle acceleration, apoptosis reduction, and promoting of cell migration. RPM, a single specific inhibitor of Akt/mTOR, signal pathway, is primarily used as an immunosuppressive agent in organ transplantation postoperatively. Recent reports have indicated that RPM can inhibit proliferation of various cancer cells, such as leukemia, breast cancer, and liver cancer cells (Mondesire et al., 2004; Récher et al., 2005; Semela et al., 2007). Its anti-tumor effect works by blocking cell cycles, but it also acts by inducing cancer cell apoptosis and autophagy, resulting in cancer cell death (Brazelton and Morris, 1996; Faivre et al., 2006). It has also been found to reduce cancer angiogenesis by decreasing HIF-1α and VEGF, activation of endothelial cell proliferation and migration, as well as by increasing thrombosis in tumor neovascularization (Young and Jan, 2006). Hudson et al. (2002) have reported that RPM can inhibit the expression of HIF-1α and its transcriptional activation effect on VEGF in prostate cancer cells *in vitro*.

Cisplatin, one of the first-line chemotherapy drugs, is mainly used for chemo radiotherapy and for advanced or recurrent cervical cancer patients. However, its non-specific side effects and drug resistance commonly limit its clinical use in advance cervical cancer patients. Yuan et al. (2003) showed that the increased activity of Akt/mTOR signal pathway in ovarian cancer and breast cancer results in cisplatin resistance. Micheal et al. (2003) and Liu et al. (2007) have drawn the same conclusion based on their research on ovarian cancer and lung cancer, respectively, suggesting that Akt/mTOR inhibitor RPM can reverse cisplatin resistance In this study, cervical cancer HeLa cells were treated with RPM alone or combined with cisplatin *in vitro* to further detect mRNA and protein expression of HIF-1α and VEGF using RT-PCR and western blot assay. Our findings demonstrated that HIF-1α and VEGF expression levels were decreased in a subtoxic dose (20 mg/ml).

Mondesire et al. (2004) in the RPM group, subtoxic dose (0.5 mg/ml) (Jiang et al., 2001) in the cisplatin group, and significantly in the combination group. The inhibitory effect of RPM combined with cisplatin on HIF-1α and VEGF expression was higher than their effect when they were used separately, suggesting that the combined usage might reduce the dosage of chemotherapy drugs.

BaeJump et al. (2009) have reported a synergistic effect of RPM and cisplatin combination on endometrial cancer cells. Wu et al. (2005) have proposed that RPM can increase the sensitivity of drug-resistant lung cancer cell lines on cisplatin, leading to cell apoptosis of resistant lines due to a synergistic effect. The synergistic effect could be explained with the inhibitory Akt/mTOR signal pathway, reversal cisplatin resistance, but also with cell apoptosis despite RPM-induced cell cycle blockage, cancer cell

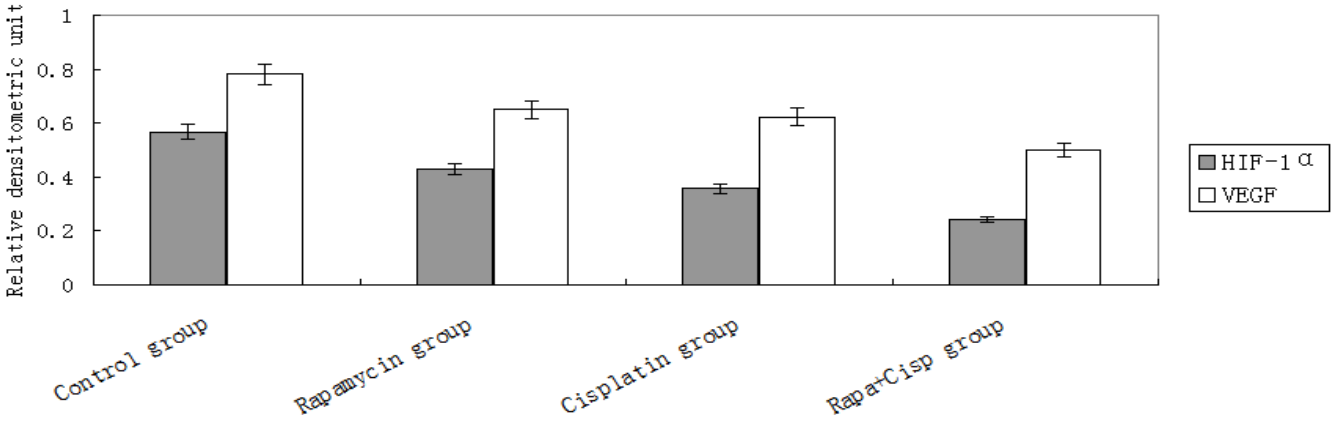

Figure 2. The relative density analysis of mRNA expression of HIF-1α and VEGF detected by RT-PCR.

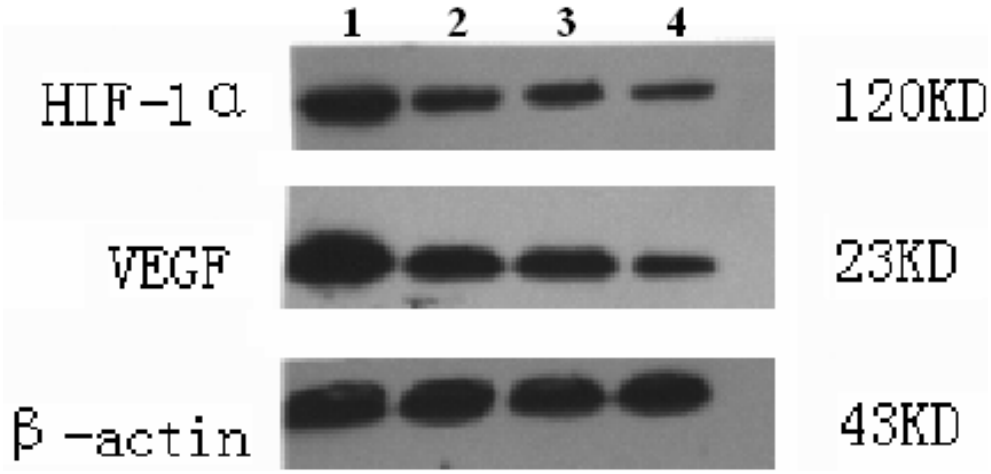

Figure 3. Protein electrophoretogram of HIF-1α and VEGF detected by western blot assay. 1, Control group; 2, RPM group; 3, cisplatin group; 4, combination group.

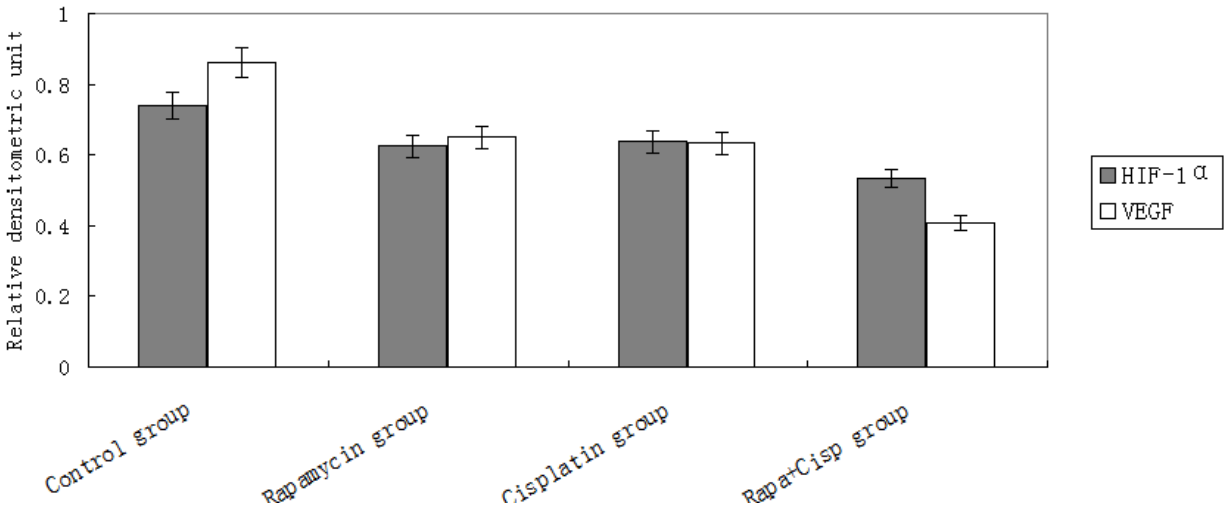

Figure 4. The relative density analysis of protein expression of HIF-1α and VEGF detected by western blot assay.

Table 1. The effects of RPM and cisplatin used alone or in combination on the mRNA expression in HeLa cells.

Group	HIF-1α mRNA	VEGF mRNA
Control	0.567 ± 0.084	0.781 ± 0.139
Rapamycin	0.428 ± 0.068*	0.651 ± 0.112*
Cisplatin	0.357 ± 0.051*	0.623 ± 0.125*
Combination	0.242 ± 0.048*△	0.498 ± 0.093*△

*p < 0.05, versus Control group; △p < 0.05, versus RPM and cisplatin groups.

Table 2. The effect of every group on expression levels of HIF-1α and VEGF protein in the Hela cells.

Group	HIF-1α IOD	VEGF IOD
Control	0.739 ± 0.159	0.861 ± 0.161
Rapamycin	0.625 ± 0.132*	0.650 ± 0.114*
Cisplatin	0.635 ± 0.120*	0.623 ± 0.102*
Combination	0.514 ± 0.092*△	0.409 ± 0.082*△

*p < 0.05, versus control group; △p < 0.05, versus RPM and cisplatin groups.

apoptosis and autophagy, as well as with reduced cancer angiogenesis by decreasing HIF-1α and VEGF. Further research and studies on the synergistic anti-tumor effect of RPM and cisplatin are necessary.

A possibility of RPM to kill cancer cells with high selectivity without attacking normal cells (Podsypanina et al., 2001) has also been suggested. The synergistic effect between RPM and chemotherapy drugs can reduce drug dose, thus, decreasing the side effect of traditional chemotherapy and enhancing the patient's quality of life. In a study on the influence of the combination of RPM and taxol on subcutaneous xenograft ovarian cancer in nude mice, Jiang and Feng (2006) have found decreased micro vessel density and lower expression levels of both HIF-1α and VEGF. Similarly, the combination of RPM and imatinib in chronic myeloid leukemia has enhanced survival in nude mice (Mohi et al., 2004). In this way, the combination of RPM and anti-tumor drugs would probably provide a novel anti-tumor option for the treatment of malignancies.

REFERENCES

Aleskog A, Norberg M, Nygren P, Rickardson L, Kanduri M, Tobin G, Aberg M, Gustafsson MG, Rosenquist R, Lindhagen E (2008). Rapamycin shows anticancer activity in primary chronic lymphocytic leukemia cells in vitro, as single agent and in droog combination. Leuk Lymphoma 49:2333-2343.

Bachtiary B, Schindl M, Potter R (2003). Over expression of hypoxia-inducible factor 1alpha indicates diminished response to radiotherapy and unfavorable prognosis in patients receiving radical radiotherapy for cervical cancer. Clin. Cancer Res. 9:2234-2240.

BaeJump VL, Zhou C, Boggess JF, Gehrig PA (2009). Synergistic effect of rapamycin and cisplatin in endometrial cancer cells. Cancer 115:3887-3896.

Blagosklonny MV (2001). Hypoxia-inducible factor: zchilles heel of anti angiogenic. Cancer Therapy. Int. J. Oncol. 19(2):257-262.

Bos R, Zhong H, Hanrahan CF (2001). Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J. Natl. Cancer Inst. 93:309-314.

Brazelton TR, Morris RE (1996). Molecular mechanisms of action of new xenobiotic immunosuppressive drugs: tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. Curr. Opin. Immunol. 8:710-720.

Burri P, Djonov V, Aebersold DM (2003). Significant correlation of hypoxia-inducible factor-1alpha with treatment outcome in cervical cancer treated with radical radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 56:494-501.

Carmeliet P, Ferreira V, Bieier G (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 380:435-439.

Faivre S, Kroemer G, Raumond E (2006). Current development of mTOR inhibitors as anticancer agents. Nat. Rev. Drug Discov. 5:671-688.

Hidalgo M, Rowinsky EK (2000). The rapamycin-sensitive signal transduce pathway as a targ for cancer therapy. Oncogene. 19:6680-6686.

Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, Giaccia AJ, Abraham RT (2002). Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. Mol. Cell. Biol. 22:7004-7014.

Jiang H, Feng Y (2006). Hypoxia-inducible factor-1alpha (HIF-1alpha) correlated with tumor growth and apoptosis in ovarian cancer. Int. J. Gynecol. Cancer 16:405-412.

Jiang YH, Cheng YF(2001). Study of low dose cisplatin with radiotherapy enhancing the experimental human cervical carcinoma passage cells sensitivity produced by radiation. Prog. J. Obstet Gynecol. 3(10):106-107

Liu LZ, Zhou XZ, Qian G, Shi X, Fang J, Jiang BH (2007). AKT1 amplification regulates cisplatin resistance in human lung cancer cells through the mammalian target of rapamycin/p70S6K1 pathway. Cancer Res. 67:6325-6332.

Michael F, Brendan ML, Yan XJ, Dan HC, Cheng JQ, Tsang BK (2003). p53 is a determinant of X-linked inhibitor of apoptosis protein/Akt-mediated chemo resistance in human ovarian cancer cells. Cancer Res. 63:7081-7088.

Minet E, Michel G, Remacle J, Michiels C (2000). Role of HIF-1 as a transcription factor involved in embryonic development, cancer progression and apoptosis. Int. J. Mol. Med. 5:253-259.

Mohi MG, Boulton C, Gu TL, Sternberg DW, Neuberg D, Griffin JD, Gilliland DG, Neel BG (2004). Combination of rapamycin and protein tyrosine (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. PNAS. 101:3130-3105.

Mondesire WH, Jian W, Zhang H, Ensor J, Hung MC, Mills GB, Meric-Bernstam F (2004). Targeting mammalian target of rapamycin synergistically enhances chemotherapy-induced cytotoxicity in breast cancer cells. Clin. Cancer Res. 10:7031-7042.

Podsypanina K, Lee R T, Politis C, Hennessy I, Crane A, Puc J, Neshat M, Wang H, Yang L, Gibbons J, Frost P, Dreisbach V, Blenis J, Gaciong Z, Fisher P, Sawyers C, Hedrick-Ellenson L, Parsons R (2001). An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in PTEN+/-mice. Proc. Natl. Acad. Sci. USA. 98:10320-10325.

Récher C, Beyne-Rauzy O, Demur C, Chicanne G, Dos Santos C, Mas VM, Benzaquen D, Laurent G, Huguet F, Payrastre B (2005). Antileukemic activity of rapamycin in acute myeloid leukemia. Blood 105:2517-2534.

Semela D, Piguet AC, Koiev M, Schmitter K, Hlushchuk R, Djonov V, Stoupis C, Dufour JF (2007). Vascular remodeling and antitumoral effects of mTOR inhibition in a rat model of hepatocellular carcinoma. J. Hepatol. 46:840-848.

Sivridis E, Giatromanolaki A, Gatter KC, Harris AL, Koukourakis MI (2002). Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer 95:1055-1063.

Sonode T, Kobayashi H, Kaku T (2003). Expression of angiogenesis factors in monolayer culture, multicellular spheroid and in vivo transplanted tumor by human ovarian cancer cell lines. Cancer Lett. 196:229-237.

Wong CH, Theresa L, Karen H (2003). VEGF and HIF-1α expression are increased in advanced stages of epithelial ovarian cancer. Gynecol. Oncol. 91:513-517.

Wu C, Wangpaichitr M, Feun L, Kuo MT, Robles C, Lampidis T, Savaraj N (2005). Overcoming cisplatin resistance by mTOR inhibitor in lung cancer. Mol. Cancer 4:25.

Young SK, Jan CK (2006). Inhibition of co meal neovascularization by rapamycin. Exp. Mol. Med. 38:173-179.

Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ (2003). AKT2 inhibition of cisplatin induced JNK/p38 and Bax activation by phosphorylation of ASK1. J. Biol. Chem. 278:23432-23440.