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Treating triple-negative breast cancer by a combination of rapamycin and cyclophosphamide: An *in vivo* bioluminescence imaging study

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ABSTRACT

Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, has been shown to inhibit the growth of oestrogen positive breast cancer. However, triple-negative (TN) breast cancer is resistant to rapamycin treatment *in vitro*. We set to test a combination treatment of rapamycin with DNA-damage agent, cyclophosphamide, in a TN breast cancer model. By binding to and disrupting cellular DNA, cyclophosphamide kills cells via interfering with their normal functions. We assessed the responses of nude mice bearing tumour xenografts of TN MDA-MB-231 cells to the combination of rapamycin and cyclophosphamide in both orthotopic mammary and lung-metastasis models. We tracked tumour growth and metastasis by bioluminescent imaging and examined the expression of Ki67, CD34 and HIF-1α in tumour tissues by immunohistochemistry and apoptosis index with TUNEL assay, and found that MDA-MB-231 cells are sensitive to rapamycin therapy in orthotopic mammary, but not in lung with metastasis. Rapamycin when combined with cyclophosphamide is found to have a more significant effect in reducing tumour volume and metastasis with a much improved survival rate. Our data also show that the sensitivity of TN tumours to rapamycin is associated with the microenvironment of the tumour cells. The data indicate that in a relatively hypoxic environment HIF-1α may play a role in mediating the anti-cancer effect of rapamycin and cyclophosphamide may prevent the feedback activation of Akt by rapamycin. Overall our results show that rapamycin plus cyclophosphamide can achieve an improved efficacy in suppressing tumour growth and metastasis, suggesting that the combination therapy can be a promising treatment option for TN cancer.

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1. Introduction

Triple-negative (TN) breast cancer, which is ER-negative, PR-negative and HER2 overexpression-negative, relapses quickly in response to clinical treatment[1] as this subtype of breast cancer has a high histological grade and a poor prognosis.[2] Patients with TN breast cancer, accounting for about 10–17% of all breast cancer cases,[3] are often unresponsive to endocrine agents such as trastuzumab and less responsive to standard adjuvant therapy.[4] Metastasis of TN breast cancer is often aggressive[1] and currently there is no recommended chemotherapy for the patients.[5] Some of the promising treatment regimens may consist of chemotherapy drugs and inhibitors of the mammalian target of rapamycin (mTOR) since the mTOR pathway is downstream of proto-oncogenes like Ras, PI3K, Akt, Rheb, eIF4E and Cyclin D1 as well as tumour suppressor genes such as PTEN, TSC1/2 and p53.[6]

Aberrant activation of the PI3K/Akt/mTOR pathway is involved in the oncogenesis and progression of breast cancer.[7] mTOR inhibitors, rapamycin and its analogs such as CCI-779, RAD001 and AP23573 have been introduced into clinical trials as anti-cancer agents. These agents generally have shown well-tolerable safety profiles and a promising anti-tumour effect in several types of refractory tumours.[8–10] However, clinical trials have shown only modest responses in 7–30% of cancer patients.[11] *In vitro* study shows that the TN MDA-MB-231 cells are resistant to growth inhibition induced by rapamycin.[12] Thomas and colleagues demonstrated that factors like HIF-1α level determine the sensitivity of kidney cancer cells to inhibition of mTOR (CCI-779).[13]

Several research findings suggest that the combination of rapamycin, a cytostatic agent, with DNA-damage agents could be optimal for solid tumour therapy. It has been shown that rapamycin can enhance carboplatin-induced cytotoxicity in HER2-overexpressing breast cancer cells[14] and mTOR inhibitor RAD001 can enhance the effect of cisplatin-induced apoptosis through inhibition of p21 translation.[15] Cyclophosphamide, an alkylating agent widely used in breast cancer therapy, causes DNA adducts, strand breaks and crosslink by binding to nucleophilic sites.[16] A combination of RAD001 with cyclophosphamide has shown synergistic anti-tumour activity in a gastric cancer mouse model.[17]

In this study we aim to determine whether a combination of rapamycin and cyclophosphamide has a potential improved anti-tumour effect on TN breast cancer cells (MDA-MB231) and whether the microenvironment of tumour growth has an impact on the sensitivity and resistance of TN tumour cells to mono- or combination therapy of rapamycin through HIF-1α. We first developed mouse xenograft tumour with luciferase-labelled cell line MDA-MB-231 as a preclinical TN breast cancer model and then investigated the response of the TN breast cancer to both therapeutic strategies *in vivo* via bioluminescence imaging and immunohistochemistry.

2. Materials and methods

2.1. Animal models

Female nude mice at the age of 6–8 weeks were purchased (Harlan Laboratory) and housed under standard conditions, fed with standard pelleted rodent chow and kept in a 12-h light/12-h dark cycle. All experimental procedures involving animals were approved by the institutional IRB. Prior to all surgical procedures, the animals were anesthetised with ketamine (100 mg/kg) and xylazine (15 mg/kg), according to the approved animal experiment protocol.

MDA-MB-231-luc-D3H2LN cell line stably expressing firefly luciferase was purchased from Caliper Life Sciences (Hopkinton, MA). The cells were cultured in Eagle's Minimum Essential Medium (ATCC, containing non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 1500 mg/l sodium bicarbonate) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and grown in a 5% CO_2 incubator at 37 °C before use. Orthotopic breast tumours were established by injecting 2×10^6 MDA-MB-231 cells (in 100 μl of PBS) into the fat pad of mouse mammary glands. Tumour volume was monitored weekly by measuring two perpendicular tumour diameters with a caliper and was calculated by the formula: tumour volume [mm^3] = (length [mm]) × (width [mm])^2 × 0.52. A lung-metastasis breast cancer model was established by injecting 1×10^5 MDA-MB-231 cells (in 100 μl of PBS) via tail veins of the mice. Mouse body weight was recorded weekly.

2.2. Drug preparation and treatment

Rapamycin was purchased from LC Laboratories (Woburn, MA). It was dissolved in DMSO (100 mg/ml) and was kept at −20 °C. Store solution was diluted with 1 × PBS to 1 mg/ml before use. Cyclophosphamide (Cytoxan®, Bristol-Myers Squibb Company) was directly dissolved in 1 × PBS (30 mg/ml). Both the working solutions were kept at 4 °C.

After implantation into the mice, the tumour cells were allowed to grow for 3 d without any treatment. Mice with orthotopic breast tumours were randomly divided into six groups: (1) vehicle (1% DMSO in PBS) group (control), (2) high-dose rapamycin-only group (Hi-Rapa), (3) low-dose rapamycin-only group (Lo-Rapa), (4) cyclophosphamide-only group (Cyclo), (5) combination group 1 (Hi-Rapa + Cyclo) and (6) combination group 2 (Lo-Rapa + Cyclo). There were 6–11 mice per group. For Hi-Rapa, Lo-Rapa and Hi- and Lo-Rapa + Cyclo groups, rapamycin treatment was administrated intraperitoneally (i.p.) at 5 mg/kg for the high-dose group and 1.5 mg/kg for the low-dose group. Rapamycin was given for 6 consecutive days, stopped for 1 d and then repeated for four cycles. For Cyclo and Rapa + Cyclo groups, cyclophosphamide was given by i.p. at 150 mg/kg on day 1, 3, 5, followed by a 2-week break, and the above-mentioned procedure was repeated. The mice were treated for 4 weeks in total. For the mice that were implanted with tumour cells via tail vein injection, they were randomly divided into four groups: (1) control group, (2) Hi-Rapa group, (3) Cyclo group and (4) Hi-Rapa + Cyclo group. There were five mice per group. Drug treatment regimen was the same as described above for orthotopic tumour-bearing mice, but lasted for 8–14 weeks. At the end of the experiments, we harvested the tumour samples for histology.

2.3. Cell cytotoxicity assay

Cytotoxicity tests were performed using the Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS) assay to