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Original Article

Role and mechanism of TRPS1 gene in multidrug-resistance of osteosarcoma

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Abstract: The study objective was to investigate the effect of trichorhinophalangeal syndrome 1 (TRPS1) gene expression on multidrug-resistance of osteosarcoma and to reveal the mechanism. PCR and Western Blot were performed to determine the expression of TRPS1 and multidrug resistance 1 (MDR1) in U2-OS, MG-63, Saos-2 and U2-OS-siTRPS1 (U2-OS cells with inhibited expression of TRPS1) cell lines. Immunohistochemistry was used to determine the correlation between TRPS1 and MDR1 expressions in 63 cases of osteosarcoma tissues. Cholecystokinin-8 (CCK-8) assay was performed to compare the chemotherapeutic drug-resistance of U2-OS and U2-OS-siTRPS1 cells. TRPS1 and MDR1 were most highly expressed in U2-OS cells, and less expressed in MG-63 cells. There were no P-gp proteins detected in Saos-2 cells. Correlation analysis showed that the expressions of TRPS1 and MDR1 in osteosarcoma cell lines were significantly correlated ($P<0.001$). Immunohistochemical staining of human osteosarcoma tissues showed that the expression of TRPS1 was positively correlated with the expression of P-gp in human osteosarcoma tissues ($R^2=0.9658$). siRNA mediated inhibition of TRPS1 in U2-OS cells resulted in significantly down-regulated expression of MDR1 ($P<0.05$) as well as significantly decreased the resistance to chemotherapeutic drugs such as adriamycin, methotrexate, paclitaxel and carboplatin (RI<0.5). The TRPS1 gene modulates drug-resistance of osteosarcoma cells by regulating the multidrug-resistance gene (MDR1) of osteosarcoma. Inhibition of TRPS1 expression could reduce the resistance to chemotherapeutic drugs of osteosarcoma cells.

Keywords: Osteosarcoma, multidrug-resistance, TRPS1, the multidrug-resistance gene of osteosarcoma

Introduction

Osteosarcoma (OS) is one of the most common primary malignant tumors among 10-20 year olds [1] with the incidence of about $400/10^7$ in the world. The incidence of male is about 1.5 times that of female, and the location of the disease is mainly around the knee joint [2-3]. Surgical resection and chemotherapy are the two most commonly used treatments for patients with osteosarcoma. Although the 5 year survival rate is only 20% for the patients treated with surgical resection alone, it can be improve to 60%-70% [4] by adding chemotherapy prior to surgical resections thanks to the development of multiple chemotherapeutic drugs such as methotrexate, doxorubicin, cisplatin, carboplatin and paclitaxel as well as the invention of a variety of chemotherapy regimens. Therefore, it is of great importance to treat the osteosarcoma patients with chemotherapy in order to improve their prognosis and survival rates. However, despite the continuous progress in medical technologies as well as the development of new chemotherapy methods to treat osteosarcoma in recent years, the 5 year survival rate remains at between 60%-70%, which has not been improved significantly. One of the important reasons for the failure to improve the survival rate of osteosarcoma patients is that the pathogenesis of osteosarcoma has not yet been revealed [5]. Multidrug-resistance emerged in the process of chemotherapy for patients with osteosarcoma is another main factor that affects the efficacy of chemotherapy as well as the survival rate of OS patients [6-7]. Therefore, it is crucial to study the occurrence and regulation mechanism of multidrug-resistance in OS chemotherapy to improve the clinical efficacy and survival rate of OS patients.

Table 1. RT-PCR primers for TRPS1, Mdr-1 and β-actin

Gene	Primer sequence (5'-3')	Length (bp)
TRPS1	F: TCAATGGCCACAGGTCAAAGA	493
	R: GAGGGCCTTCCACTAAACCC	
Mdr-1	F: GGGTATGGTTAGCATGCGAGT	389
	R: GGAAACTAAATGCAGCCCAGTT	
β-actin	F: AAGTACTCCGTGTGGATCGG	615
	R: TCAAGTTGGGGGACAAAAAG	

Trichorhinophalangeal syndrome 1 (TRPS1) is one of the genes that play an important role in regulating chondrocyte proliferation and apoptosis [8]. Multiple studies around the world [9-11] have shown that TRPS1 is generally over expressed in breast cancer, colon cancer as well as prostate cancer, which is probably related to tumor cells invasion/metastasis and the formation of multidrug-resistance during chemotherapy. In this study, we compared the relationship of TRPS1 expression and the expression of the multidrug-resistance gene multidrug-resistance 1 (MDR1) in different osteosarcoma cell lines as well as in human osteosarcoma tissues, and revealed the role of TRPS1 gene in multidrug-resistance of osteosarcoma by the combined technology of siRNA interfering and drug sensitivity test.

Materials and methods

Specimen sources

63 cases of surgically resected osteosarcoma tissues were collected from diagnosed osteosarcoma patients (Inclusion criteria: first, the patients should not be associated with other tumors; second, the clinical symptoms of the patients were not obviously improved after 1-2 cycles of chemotherapy; third, the imaging detection of tumor progression or the postoperative pathological examination of tumor necrosis rate should be less than 80%) in the Third Affiliated Hospital of Kunming Medical University from January 2013 to December 2015). The tissue samples were fixed in formalin and embedded in paraffin before they were made into tissue sections and stored at 4°C. Among the 63 patients, there were 39 cases of male patients and 24 cases of female patients. They were aged 14-25 years, with the mean age of (18.3+5.2) years old. None of the patients received any chemotherapy or radiotherapy treatment before the surgical resection of the diseased tissues. The study methods used in this research were approved by the ethics committee of the Third Affiliated Hospital of Kunming Medical University, and all the patients have signed informed consent in the written form.

Experimental materials

U2-OS, MG-63 and Saos-2 osteosarcoma cell lines were purchased from ATCC (American Culture Preservation Center, Maryland, USA) (cells were cultured according to the ATCC guidelines); the TRPS1 expression interfering plasmid U2-OS-siTRPS1 was constructed in our lab for this experiment; TRPS1 (N-18) antibody (sc26974), P-gp antibody (sc-73354), donkey anti-goat IgG (sc-3850) and goat anti-mouse IgG (sc-3696) were purchased form Santa Cruz Biotechnology (Shanghai) Co., Ltd.; the reverse transcription kit was purchased from Fermentas (K1622, MBI company, Lithuania); the CCK-8 kit was purchased from DOJINDO (CK04-3000T, Dongren Chemical Technology (Shanghai) Co., Ltd., Shanghai, China).

Experimental methods

PCR detection of the mRNA expressions of TRPS1 and Mdr-1

U2-OS, Saos-2 osteosarcoma cells and C5-D7 cells (the same as U2-OS) were cultured according to the ATCC guidelines. When cells grew to confluence, trypsin (Gibco, 25200-056) was used to digest the cells and 10 mL culture medium was used to resuspend the cells. After centrifugation (1000 rpm, 10 min, room temperature) the culture medium was discarded and the cells were subjected to total RNA extraction by the cellular RNA extraction kit (Noob Ladd, Beijing), and then the reverse transcription kit were used to prepare cDNA. RT-PCR was performed and 10 uL PCR product of each sample was loaded for nucleic acid gel electrophoresis. The results were observed and photographed on a Bio-Rad gel imaging analyzer (Bole life medicine products Ltd, Shanghai, China). The gel imaging system Quantity One software (Bole life medicine products Co., Ltd., Shanghai, China) was used to analysis the relative content of the target gene by comparing the band of the target gene to the band of β-actin. Primers for TRPS1, Mdr-1 and

Table 2. The expression of TRPS1 and Mdr1 genes in different osteosarcoma cell lines

Groups	TRPS1		Mdr1	
	mRNA	Protein	mRNA	P-gp
U2-OS	4.55±0.21#	3.76±0.26#	2.81±0.16#	2.64±0.15#
MG-63	2.38±0.025*	1.73±0.099*	1.26±0.024*	1.25±0.062*
Saos-2	0.52±0.054*,#	0.16±0.032*,#	0.39±0.043*,#	0.00±0.00*,#

Legends: *represented significant differences compared to the U2-OS group, P<0.05; #represented significant differences compared to the MG-63 group, P<0.05.

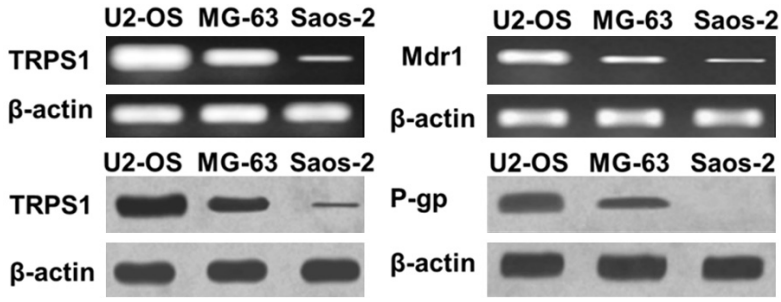

Figure 1. PCR and Western Blot detection of TRPS1 and Mdr1 expressions in different osteosarcoma cell lines.

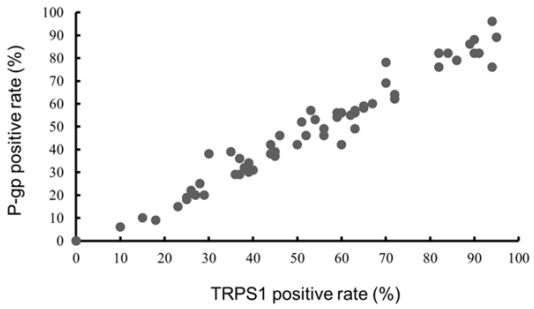

Figure 2. The correlation of TRPS1 and Mdr1 expressions in human osteosarcoma tissues.

β-actin were designed on NCBI web site, and the sequences were shown in **Table 1**.

Western Blot detection of the protein expressions of TRPS1 and Mdr-1

Cells were cultured and digested according to 1.3.1. Culture medium was discarded after centrifugation, and proteins were extracted by the total cellular protein extraction kit (Aimeijie Technologies Ltd, Shanghai, China). BCA kit (Shanghai Biyuntian Biological Technology Co., Ltd., Shanghai, China) was used to determine the protein concentrations. 50 ug protein of each sample was loaded for SDS-PAGE and transferred to a wet NC membrane. The membrane was blocked with 5% no-fat milk, incubated with primary antibodies and secondary antibodies before image development. The band density was analyzed by Image-J software and normalized against β-actin levels to be presented as the final results.

Immunohistochemistry of TRPS1 and Mdr-1 with the osteosarcoma tissue sections

20 cases of osteosarcoma tissues were subjected to immunohistochemical staining of TRPS1 and Mdr-1 (performed according to the instructions of the immunohistochemical staining kit). PBS was used as the negative control. 3 views under 400× magnification lens of each slice were randomly selected and the TRPS1 or Mdr-1 positive cells were counted to calculate the average number of positive cells.

Detection of drug-resistance indexes

The resistance index of U2-OS-siTRPS1 cells to different drugs (adriamycin, methotrexate, paclitaxel and carboplatin) was detected by CCK-8 Kit. The inhibition rate of drugs on cells= 1-OD_{600} of cells with drug treatment/OD_{600} of control cells (without drug treatment), and the median inhibitory concentration (IC50) on cells of different drugs was calculated by SPSS19.0. The drug-resistance index was calculated according to the following formula: drug-resistance index (RI) = IC50 of U2-OS-siTRPS1 cells/IC50 of U2-OS cells.

Statistical analysis methods

Statistical analysis was performed using the SPSS19.0 statistical program, measurement data was measured by (mean ± standard deviation), and t test was used to analyze the differences between groups (P<0.05 was considered statistically significant). The positive areas of immunohistochemistry staining (S values) of TRPS1 and Mdr-1 were subjected to correlation analysis.

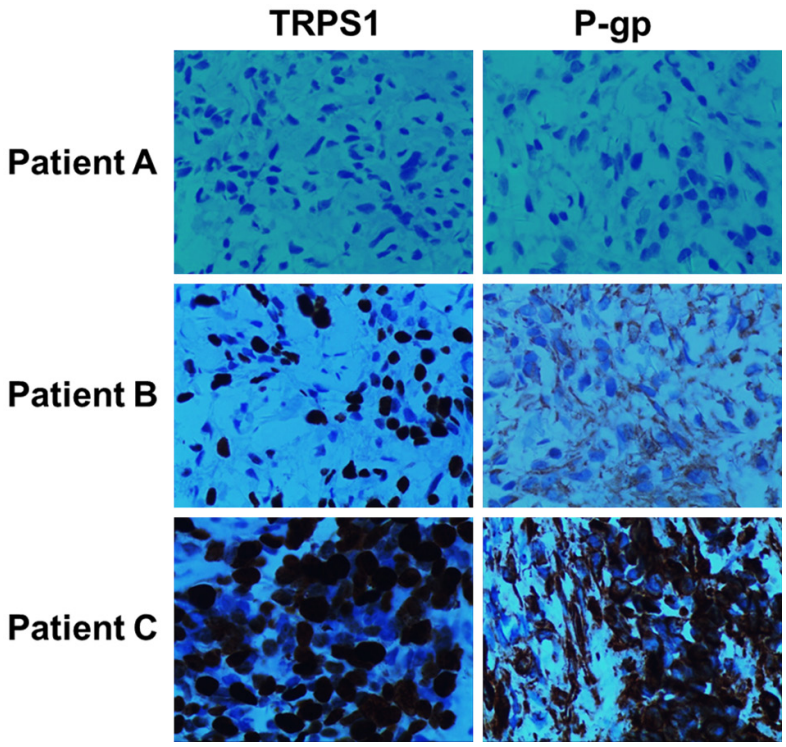

Figure 3. Immunohistochemical staining of TRPS1 and Mdr1 in the osteosarcoma tissues of different patients.

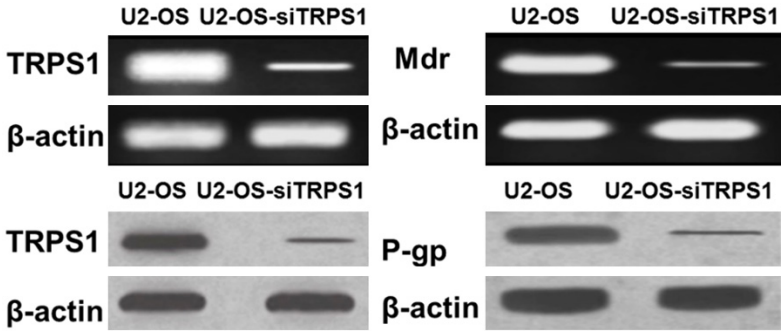

Figure 4. Effects of TRPS1 expression in OS cells on MDR1 expression.

Results

TRPS1 and Mdr1 gene expressions in different osteosarcoma cell lines

U2-OS cells showed the highest mRNA and protein expressions of TRPS1 and Mdr1 genes, which were significantly higher than those in MG-63 and Saos-2 cells (P<0.05). Saos-2 cells showed the lowest mRNA and protein expressions of TRPS1 and Mdr1 genes, which were significantly lower than those in MG-63 cells (P<0.05). No expression of P-gp protein was detected in Saos-2 cells. Moreover, Pearson correlation analysis showed that the expression of TRPS1 gene and the expression of Mdr1 gene were significantly correlated in osteosarcoma cell lines (P<0.001), as shown in **Table 2**, **Figures 1** and S1.

Immunohistochemical staining of TRPS1 and Mdr1 in different clinical osteosarcoma specimens

The correlation scatter map of TRPS1 and Mdr1 expressions in human osteosarcoma tissues were drew with the abscissa of the percentage of TRPS1 positive and the ordinate of the percentage of Mdr1 positive immunohistochemical staining of human osteosarcoma tissues (**Figure 2**). The TRPS1 positive rate was positively correlated with the P-gp positive rate in immunohistochemical staining of human osteosarcoma tissues. In addition, Pearson correlation analysis showed that there was significant correlation between the immunohistochemical staining of TRPS1 and P-gp in human osteosarcoma tissues (P< 0.001). The results of immunohistochemical staining of TRPS1 and Mdr1 in some of the human osteosarcoma tissues were shown in **Figure 3**.

The effect of TRPS1 expression on the expression of Mdr1 in osteosarcoma cell lines

After the expression of TRPS1 gene was inhibited by siRNA mediated knock-down in U2-OS cells, the Mdr1 expression was also significantly down regulated in U2-OS cells (P<0.05), as shown in **Figures 4**, S1 and **Table 3**.

The effect of TRPS1 expression on the multi-drug-resistance of osteosarcoma cell lines

The U2-OS cells with inhibited TRPS1 expression showed significantly lower drug-resistance of several chemotherapeutic drugs, such as

Table 3. Effect of TRPS1 expression on the expression of Mdr1 in osteosarcoma cell lines

Groups	TRRS1		Mdr1	
	mRNA	Protein	mRNA	P-gp
U2-OS	4.51±0.16	3.89±0.083	2.81±0.19	2.61±0.17
U2-OS-siTRPS1	0.95±0.19	0.45±0.11	0.53±0.26	0.18±0.069
t	31.510	56.273	15.930	30.043
P	<0.001	<0.001	<0.001	<0.001

Table 4. The drug-resistance index of different chemotherapeutic drugs

Chemotherapeutic drugs	IC50 (ng/ml)		RI
	U2-OS	U2-OS-siTRPS1	
adriamycin	608×10^6	297×10^6	0.488
methotrexate	15.34	5.62	0.366
Paclitaxel	8.26	0.27	0.0327
carboplatin	3.49×10^6	0.52×10^6	0.149

adriamycin, methotrexate, paclitaxel and carboplatin (RI<0.5), as shown in **Table 4**.

Discussion

MDR refers to the phenotype in which the tumor cells that have been exposed to a long term of chemotherapy and develop not only the resistance of the chemotherapeutic drug that has been used on these cells, but also the crossed resistance to a variety of different chemotherapeutic agents with different structures and functions [12]. This is the most important defense mechanism of tumor cells to protect themselves from chemotherapeutic drugs, and also one of the major causes of chemotherapy failure. Mdr1, Mdr2 and Mdr3 are three important genes in the MDR family. Mdr2 is a type of animal gene, Mdr3 has not been thoroughly studied in the formation of chemotherapy multidrug-resistance, and Mdr1 gene has been proved to be involved in the multidrug-resistance of multiple tumor cells by a number of in vitro experiments. The Mdr1 gene is located on human chromosome 7q21-1 and is widely expressed in normal human tissues as well as tumor tissues [13]. The P-gp, encoded by the Mdr1 gene, is thought to be involved in the pathogenesis, development, and the formation of multidrug-resistance of cancer. Lu D. et al. [13] analyzed 80 cases of primary ovarian cancers, 16 cases of benign ovarian epithelial tumors and 12 cases of normal ovarian tissue samples. The results revealed that P-gp was expressed in 57.5% ovarian cancer tissues, which was significantly higher than those in benign tumors and normal tissues (P<0.05), and that the mortality rate of the P-gp positive group was 2.049 times of that of the P-gp negative group while the PGP positive group was less sensitive to the chemotherapeutic drugs. In addition, the study of Li Y et al. [14] in gastric cancer showed that the clinical efficacy of postoperative chemotherapy in patients with gastric cancer is related to the expression of MDR1 gene C3435T. The related studies on the molecular mechanisms [15-17] showed that the P-gp located in the inner cellular membranes acts as an energy-dependent drug pump, which can pump drugs out of the cells using energy release from ATP hydrolysis thus reducing the intracellular drug concentrations and leading to the formation of multidrug-resistance cells. At present, most researchers believe that the multidrug resistance mediated by P-gp is the classic formation mechanism of chemotherapeutic drug-resistance of tumor cells, and that the content of P-gp in tumor tissues is a key index to evaluation of treatment efficacy of chemotherapy and the formation of multidrug-resistance [18].

In this study, we detected the expression of TRPS1 and Mdr1 genes and found that there was significant correlation between the expression of TRPS1 and Mdr1 in osteosarcoma cell lines (P<0.001). We also performed immunohistochemistry in 63 cases of osteosarcoma tissues, and the results showed that the positive staining rate of TRPS1 was positively related to the positive staining rate of P-gp in human osteosarcoma tissues (R^2=0.9658). These results suggested that TRPS1 gene was likely involved in the multidrug-resistance of osteosarcoma cell lines.

The TRPS1 gene is located on the human chromosome 8q24.1, and its encoded protein is an important member of the GATA transcription factor family. The Chinese name for TRPS1 gene means the hair, nose, and toe syndrome-1 gene, as the name suggested, it is also related to the growth and development of human hair and bones. The studies of Su P et al. [19] sug-

gested that TRPS1 can not only regulate the proliferation and apoptosis of human chondrocytes, but also regulate the expression of bone proteins through binding with the bone protein promoters. Moreover, the researches of Hong J et al. [20] further pointed out that TRPS1 mutations and deletions can cause dysplasia of face, hair and skeleton. In addition, multiple studies [10, 21, 22] have shown that TRPS1 is over expressed in breast cancer, prostatic cancer and many other solid tumors, and that it is even higher expressed in multidrug-resistant tumor cells indicating its important role in tumor genesis, development, and formation of drug-resistance.

In this study, we found that in U2-OS cells with inhibited TRPS1 expression, the expression of Mdr1 gene was also significantly inhibited, and that the P-gp protein was significantly down-regulated ($P<0.05$). We further performed drug sensitivity tests, and the results showed that inhibiting the expression of TRPS1 gene in U2-OS cells could improve the cell sensitivity to various chemotherapeutic agents such as adriamycin, methotrexate, paclitaxel and carboplatin ($RI<0.5$). Taken together, our results suggested that TRPS1 could regulate the drug-resistance of osteosarcoma cells by regulating Mdr1 gene, and that inhibition of TRPS1 expression could reduce the chemotherapy drug-resistance of osteosarcoma cells.

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Disclosure of conflict of interest

None.

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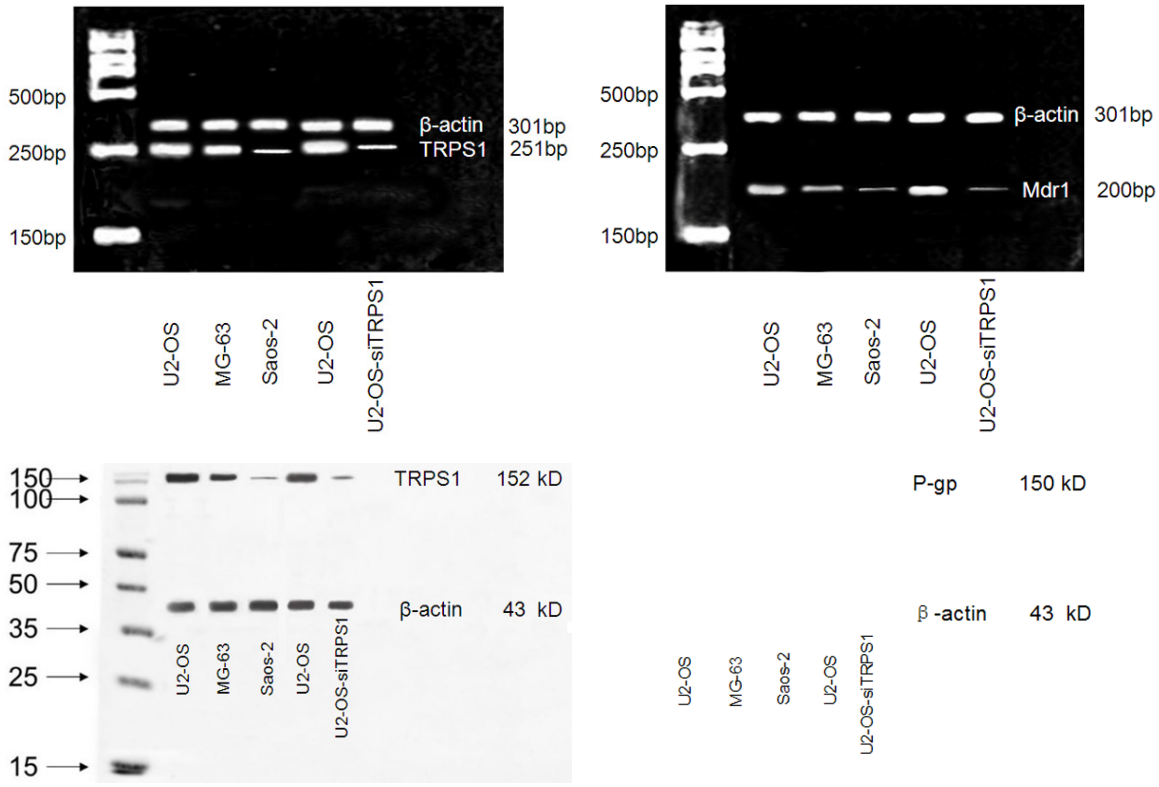

Figure S1. The original photos of PCR and Western blotting.