RESEARCH COMMUNICATION

Down-regulation of SENP1 Expression Increases Apoptosis of Burkitt Lymphoma Cells

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Abstract

Objective: **To investigate the effect of down-regulation of Sentrin/SUMO-specific protease 1 (SENP1) expression on the apoptosis of human Burkitt lymphoma cells (Daudi cells) and potential mechanisms.** **Methods:** **Short hairpin RNA (shRNA) targeting SENP1 was designed and synthesized and then cloned into a lentiviral vector. A lentiviral packaging plasmid was used to transfect Daudi cells (sh-SENP1-Daudi group). Daudi cells without transfection (Daudi group) and Daudi cells transfected with blank plasmid (sh-NC-Daudi group) served as control groups. Flow cytometry was performed to screen GFP positive cells and semiquantitative PCR and Western blot assays were employed to detect the inference efficiency. The morphology of cells was observed under a microscope before and after transfection. Fluorescence quantitative PCR and Western blot assays were conducted to measure the mRNA and protein expression of apoptosis related molecules (caspase-3, 8 and 9). After treatment with $COCl_2$ for 24 h, the mRNA and protein expression of hypoxia inducible factor -1α (HIF-1α) was determined.** **Results:** **Sequencing showed the expression vectors of shRNA targeting SENP1 to be successfully constructed. Following screening of GFP positive cells by FCM, semiqualitative PCR showed the interference efficiency was 79.2±0.026%. At 48 h after transfection, the Daudi cells became shrunken, had irregular edges and presented apoptotic bodies. Western blot assay revealed increase in expression of caspase-3, 8 and 9 with prolongation of transfection (P<0.05). Following hypoxia treatment, mRNA expression of HIF-1α remained unchanged in three groups (P>0.05) but the protein expression of HIF-1α markedly increased (P<0.05). However, in the sh-SENP1-Daudi group, the protein expression of HIF-1α remained unchanged** **Conclusion:** **SENP1-shRNA can efficiently inhibit SENP1 expression in Daudi cells. SENP1 inhibition may promote cell apoptosis. These findings suggest that SENP1 may serve as an important target in the gene therapy of Burkitts lymphoma.**

Keywords: Sentrin/SUMO-specific protease 1 - Daudi cells - cell apoptosis - hypoxia inducible factor -1α

Introduction

Burkitt lymphoma (BL) is a highly malignant B-cell non-Hodgkin's lymphoma (NHL) (Yeh et al., 2000). Currently, chemotherapy is still a major strategy in the treatment of BL. However, the chemotherapeutics not only kill the cancer cells but severely damage the normal cells and tissues. Thus, some severe side effects are frequently found during the chemotherapy. Sentrin/SUMO-specific protease 1 (SENP1) is a member of SUMO specific protease family (SENPs) and widely distributed in the nuclei. SENP1 can regulate multiple molecules related to the biological characteristics of cancers via desumoylation. Among these molecules, hypoxia induced factor-1α (HIF-1α) which is widely considered to be one of the key regulators in cancer cells is an important one regulated by SENP1 (Yeh et al., 2000; Cheng et al., 2007; Song et al., 2011). In the present study, RNA interference was employed to down-regulate SENP1 expression in Burkitt lymphoma cells (Daudi cells) and then the apoptosis was observed. Moreover, the potential role of HIF-1α in this effect was also explored. Our findings may confirm SENP1 as a new target in the gene therapy of Burkitt lymphoma.

Materials and Methods

Main reagents

pLVTHM Vector expressing shRNA (Trono Lab), RPMI 1640 medium, fetal bovine serum (Gibco), TRIzol RNA extraction kit (Invitrogen), reverse transcription kit (Promega, USA), SYBR Green real time quantitative PCR kit (TaKaRa), SENP-1 antibody and HIF-1α antibody (Abcam, USA) were used in the present study.

Design and synthesis of primers

The primers for PCR were synthesized in Shanghai

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Sangon. SENP1: 5'-TTGGCCAGAGTGCAAATGG-3' (forward), 5'-TCGGCTGTTTCTTGATTTTTGTA-3' (reverse), anticipated size: 80 bp; HIF-1α: 5'-CCGAATTGATGGGATATGAG-3' (forward), 5'-TCATGATGAGT TTTGGTCAGATG-3' (reverse), anticipated size: 220 bp.

Cell culture

Daudi cells were purchased from Cell Institute of Chinese Academy of Sciences. Lenviral packaging 293T cells were maintained in DMEM containing 10% FBS. Human Burkitt lymphoma cells (Daudi cells) were grown in RPMI 1640 containing 10% FBS. Cells were cultured at 37 °C in a humidified environment with 5% CO_2 and those in logarithmic growth phase were used for the following experiments.

Construction of lentiviral vectors expressing SENP1 shRNA, transfection and screening of Daudi cells

According to the principles for the design of RNA interference sequence, a total of 3 sequences targeting SENP1 gene (NM_014554) were designed: SH1 (5'-TTCCTTTACACCTGTCTCG-3'), SH2 (5'-CACCACTCTGTTCCACATC3'), and SH3 (5'-TCAGGCAGTGAAACGTTGG -3'). The sh1+sh2+sh3 packaging lentiviral vectors were prepared and the lentiviral packaging plasmids were used to transfect 293T cells. The viral solution was prepared and named pRNAT/shSENP1-Daudi group. At the same time, shRNA targeting scrambled sequence (general sequence: 5'-TTCTCCGAACGTGTCACGT-3') was designed and served as negative control group (pRNAT/NC). The lentivirus was labeled with green fluorescent protein (GFP). Following dilution, flow cytometry was performed to measure the proportion of GFP positive lentivirus to determine the viral titer. The lentivirus in the pRNAT/sh-SENP1-Daudi group and pRNAT/NC was used to transfect Daudi cells at appropriate titer and named sh-SENP1-Daudi and sh-NC-Daudi (negative control) groups, respectively. In addition, Daudi cells without transfection served as a blank control group. At 24 and 48 h after transfection, the GFP expression was observed in different groups. Cells with GFP positive rate of > 50% were used in the following experiments. Moreover, at 48 h after transfection, flow cytometry was performed to screen GFP positive Daudi cells for further culture and experiments (Wang et al., 2006).

Detection of transfection efficiency

Real-time fluorescence quantitative PCR and western blot assay were employed to measure the mRNA and protein expressions of SENP1 before and after transfection. At 48 h after transfection, 1×10^7 cells were collected and total RNA was extracted with TRIzol reagent. Then, 1 μl of dNTP, 1 μl of MMLV, 5 μl 5×buffer, 1 μl of Olig(dt), 0.5 μl of RNasin and 1 μl of RNA were mixed followed by addition of water to 20 μl. The mixture was incubated at 42 °C for 50 min followed by denaturation at 95 °C for 5 min and subsequent reverse transcription. Then, 0.8 μl of cDNA, 10 μl of Syber green PremixII, 0.4 μl of Primer 1 (10 μM), 0.4 μl of Primer 2 (10 μM) and water were mixed at a final volume of 20 μl. PCR was performed in Roche thermal cycler. The PCR conditions were: pre-denaturation at 95°C for 1 min, 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 20 s. β-actin served as an internal reference and $2^{-\triangle\triangle CT}$ method was employed to determine the relative expression of target genes. Cell culture was identical to those above, and cells were collected 48 h later. Total protein was extracted with a kit and subjected to SDS-PAGE. Then, the proteins were transferred onto PVDF membrane which was treated with SENP1 or β-actin antibodies (1:500) and horseradish peroxidase conjugated IgG. Following visualization, bands were scanned into a computer followed by analysis of optical density. Experiment was performed three times.

RNA interference of SENP-1 expression on apoptosis of Daudi cells

At 24 h, 48 h and 72 h after transfection, total RNA was extracted with TRizol reagent and reverse transcribed into cDNA. Real-time fluorescence quantitative PCR was done to measure the mRNA expression of caspase-3, 8 and 9 in cells of sh-SENP1-Daudi group. At the same time points, total proteins were extracted and western blot assay was employed to measure the protein expression of caspase-3, 8 and 9 in cells of sh-SENP1-Daudi group. The above experiments were done three times.

RNA interference of SENP-1 expression on HIF-1α expression in Daudi cells

$CoCl_2$ (100 mmol/L) was used to mimic the hypoxic environment and treat cells in the blank control group, sh-NC-Daudi group and sh-SENP1-Daudi group for 24 h. Real-time quantitative PCR and western blot assay were performed to measure the mRNA and protein expression of HIF-1α.

Statistical analysis

Statistical analysis was performed by using SPSS 15.0 Software. The comparisons among different group were done with Chi-square test. $P < 0.05$ was considered significantly different.

Results

Daudi cells experiencing transfection

Peripheral blood lymphcytes (PBL) were separated by Percoll density gradient centrifugation in 10 healthy volunteers. Fluorescence quantitative PCR was performed to measure the mRNA expression of SENP-1 in Daudi cells. Results showed the mRNA expression of Daudi in Daudi cells was 27.83±6.64 higher than that in normal cells (P<0.05). The NC-shRNA and SENP1-shRNA lentiviral vectors were used to transfect Daudi cells at MOI of 15 for 48 h followed by laser scanning confocal microscopy. Results showed the transfection efficiency was > 50% and these cells can be used for further experiments (Figure 1).

Detection of interference efficiency

At 48 h after transfection, flow cytometry was performed to screen the GFP positive cells (Figure 2A). Quantitative PCR showed, when compared with sh-NC-
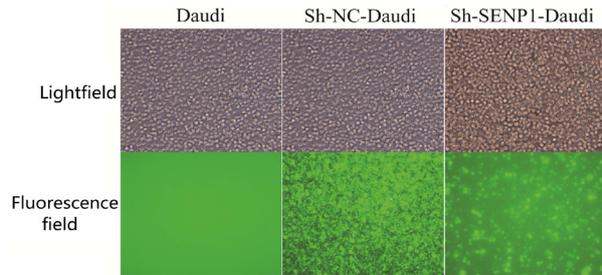
Figure 1. Microscopy of Daudi Cells after Transfection with Respective Vectors. Lentiviral vectors in the pRNAT/sh-SENP1-Daudi group and pRNAT/NC group were used to transfect Daudi cells at appropriate titer and named sh-SENP1-Daudi group and sh-NC-Daudi group (negative control group), respectively. At the same time, Daudi cells without transfection served as a blank control group. At 48 h after transfection, the GFP expression was observed in sh-SENP1-Daudi group and sh-NC-Daudi group
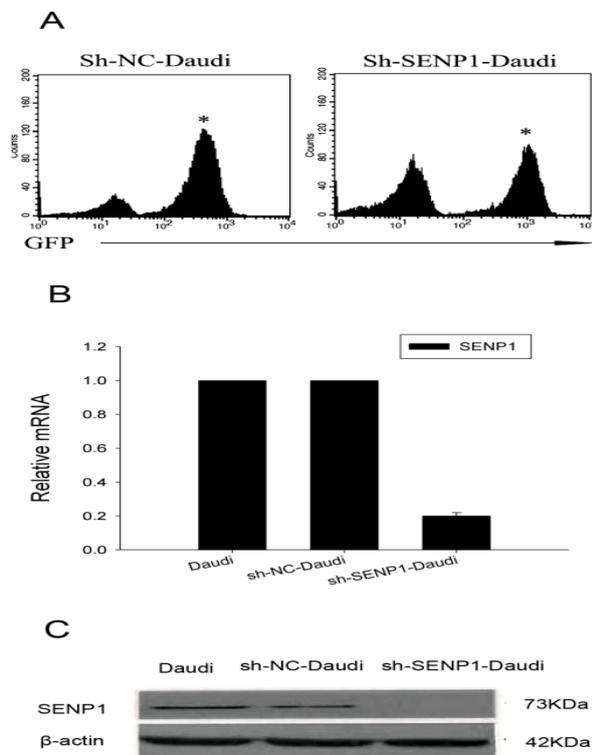
Figure 2. SENP1 Gene Down-regulation in Daudi cells. A: screening of GFP positive cells by flow cytometry; *: GFP positive cells which accounted for 81.3% in the sh-NC-Daudi grop and 57.9% in the sh-SENP1-Daudi group. B: SENP1 mRNA expression in Daudi cells transfected with lentiviral vectors expression sh-SENP1 for 48 h. C: SENP1 protein expression in Daudi cells transfected with lentiviral vectors expression sh-SENP1 for 48 h (western blot assay)

Daudi group and blank control group, the inhibition rate of SENP1 mRNA expression was $79.17\pm0.026\%$ in the sh-SENP1-Daudi group. Western blot assay revealed the protein expression of SENP1 in the sh-SENP1-Daudi group was markedly reduced as compared to the remaining two groups but there was no significant difference in the protein expression of SENP1 between sh-NC-Daudi group and blank control group (Figure 2B, 2C).

SENP1-shRNA transfection on morphology of Daudi cells
    At 48 h after transfection, flow cytometry was
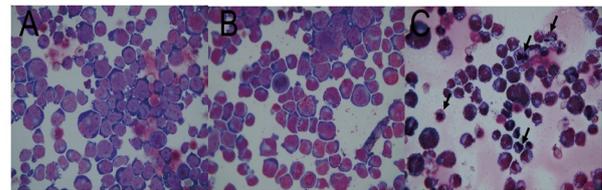
Figure 3. Giemsa Staining of Sections and Observation at 100×. A: Blank control group, B: Sh-NC-Daudi group; C: sh-SENP1-Daudi group. Arrow shows cell shrinkage, irregular cell membrane, pyknosis, nuclear fragmentation and apoptotic bodies
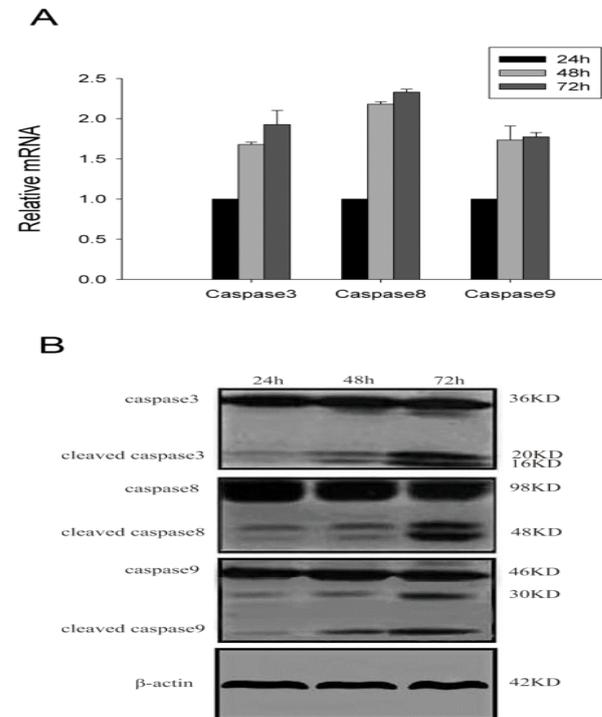
Figure 4. Effect of SENP1 Down-regulation on mRNA and Protein Expressions of Caspase-3, 8 and 9 in Daudi Cells. A: mRNA expression of caspase-3, 8 and 9 in Daudi cells undergoing sh-SENP1 transfection at 48 h and 72 h after transfection. At 24 h, 48 h and 72 h after transfection, the mRNA expression of caspase-3, 8 and 9 was increased by 1.679 ± 0.036 and 1.788 ± 0.032, 2.184 ± 0.034 and 2.369 ± 0.040 and 1.700 ± 0.025 and 1.793 ± 0.037, respectively, when compared with that in the blank control group. B: Protein expression of caspase-3, 8 and 9 in Daudi cells undergoing sh-SENP1 transfection at 48 h and 72 h after transfection

performed to screen GFP positive cells (Daudi cells without transfection did not experience screening). Following fixation, Giemsa staining of these cells were done and the morphology was observed under a light microscope (100×). Results showed cells in the sh-SENP1-Daudi were shrunk, had irregular edge in the membrane, pyknosis and nuclear fragmentation and apoptotic bodies were also noted in these cells. In the blank control group and Sh-NC-Daudi group, cells had clear edge in the membrane and no or extremely few apoptotic bodies (Figure 3)

Effect of SENP1 down-regulation on mRNA and protein expressions of caspase-3, 8 and 9 in Daudi cells
    At 24 h, 48 h and 72 h after transfection, FCM was done to screen GFP positive cells (data were not shown).

A

B
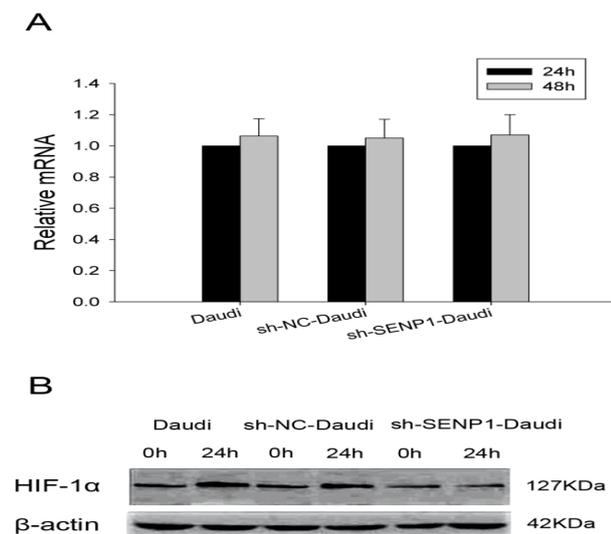
Figure 5. Effect of RNA Interference of SENP1 on mRNA and Protein Expression of HIF-1α in Daudi Cells. A: mRNA expression of HIF-1α following hypoxia treatment. Cells in the blank control group, sh-NC-Daudi group and sh-SENP1-Daudi group were treated with CoCl2 at 100 mmol/L for 24 h. Results showed the mRNA expression of HIF-1α was 1.076±0.110, 1.054±0.120 and 1.072±0.131 in the blank control group, sh-NC-Daudi group and sh-SENP1-Daudi group, respectively, showing no marked difference (P>0.05). B: Protein expression of HIF-1α following hypoxia treatment (Western blot)

Quantitative PCR and Western blot assay showed the mRNA and protein expressions of caspase-3, 8 and 9 increased with the prolongation of transfection (P<0.05) (Figure 4A and 4B).

Effect of RNA interference of SENP1 on mRNA and protein expression of HIF-1α in Daudi cells

CoCl$_2$ at 100 mmol/L was used to mimic hypoxic environment and treat cells in blank control group, Sh-NC-Daudi group and Sh-SENP1-Daudi group for 24 h. Fluorescence quantitative PCR and Western blot assay were employed to measure the mRNA and protein expression HIF-1α at 0 h and 24 h after CoCl$_2$ treatment. Results showed the mRNA expression of HIF-1α remained unchaged in three groups (P>0.05) (Figure 5A). However, after CoCl$_2$ treatment for 24 h, the protein expression of HIF-1α in the blank control group and Sh-NC-Daudi group was markedly increased but that in the Sh-SENP-1-Daudi group remained unchanged (Figure 5B).

Discussion

BL is a highly malignant tumor and usually progresses rapidly. Patients with BL have high mortality. The prevalence of BL is about 40% in Children with NHL and 2% in adults with NHL (Shen et al., 2003). With the development in molecular biology, immunohistochemistry and cytogenetics, most patients with BL may recover after high dose chemotherapy. However, after repeated chemotherapy, cancer cells may combat with the chemotherapeutics induced apoptosis and thus, a fraction of patients are non-responsive to treatment or develop

recurrence. Thus, to find the factors related to cancer cells escaping apoptosis and to explore these factors as therapeutic target may provide new strategy for the treatment of BL.

SENPs mediated desumoylation has been a hot topic in the studies on post-translational modification (Yeh et al., 2000). In recent years, abnormal SENP1 expression has been found in some cancers. For example, in human prostate cancer and thyroid oncocytoma, the protein expression of SENP1 was found to be up-regulated and the SENP1 expression was related to the invasiveness of cancer cells (Shalizi et al., 2006; Bawa-Khalfe et al., 2007). Over-expression of SENP1 was found to induce canceration of prostate epithelial cells in mice, and silencing LNCaP SENP1 expression by RNA interference in prostate cancer cell line was demonstrated to increase the spontaneous apoptosis rate and the sensitivity of prostate cancer cells to chemotherapeutics.

SENP1 can regulate multiple factors related to the biological behaviors of cancer cells among which HIF-1α is an important one. HIF-1 is a widely distributed transcriptional factor in mammalians and humans in the presence of hypoxia. HIF-1 is the heterodimer of α unit and β unit. HIF-1α is a unique subunit regulated by oxygen and oxygen determines the HIF1 activity. The Lysine residues at 391 and 477 are the site modified by SUMO-1. Following binding to target genes, HIF1 can increase the transcription of down-stream genes (vascular endothelial growth factor and multidrug resistance gene) via transcription and post-transcriptional regulation. This may alter the signal transduction of cell survival and apoptosis which plays an important role in the adaptation to hypoxia and invasion and metastasis of cancers (Yates et al., 2008). SENP1 can regulate the stability of HIF-1α to exert a series of biological effects involving in survival and immune escaping of cancer cells (Yates et al., 2008). Currently, there is controversy on the effect of SUMO on HIF-1α stability. Bae et al. (2004) found that SUMO-1 can compete with ubiquitin to bind to the same sites, which increases the HIF-1α stability. However, Cheng et al. (2007) found the HIF-1α stability reduced in mice with SENP1 deficiency, which suggests that the desumoylation of HIF-1α by SENP1 is essential for the HIF-1α stability. Thus, in the occurrence and development of cancers, the relationship between SENP1 and HIF-1α is required to be further elucidated. This elicits the interest on the role of SENP1 in the occurrence and development of cancers.

In the present study, we for the first time confirmed that the SENP1 expression in Daudi cells was higher than that in normal peripheral blood lymphocytes. This suggests SENP1 is closely related to the Burkit lymphoma cells. Then, RNA interference was employed to down-regulate SENP1 expression which was demonstrated by Western blot assay. Microscopy showed Daudi cells with SENP1 down-regulation had characteristics of apoptosis such as cell shrinkage, nuclear fragmentation and apoptotic bodies. In addition, the classic apoptotic factors caspase-3, 8 and 9 were up-regulated at mRNA and protein levels. This suggests SENP1 down-regulation increases the apoptosis of Daudi cells. Whether this process has involvement of HIF-1α stability is still unclear (Chavez et al., 2002; Jean,

2004; Resar et al., 2005; Zhou et al., 2011). In this study, simulated hypoxia was employed and results showed the mRNA expression of HIF-1α was comparable in normal Daudi cells and those with SENP1 down-regulation, but the protein expression of HIF-1α was markedly different between them. This indicates SENP1 can regulate the protein expression of HIF-1α. The hypoxia interrupts the balance between generation and degradation of HIF-1α protein, and increase the protein expression of HIF-1α. In Daudi cells with SENP1 down-regulation, the HIF-1α expression remained unchanged. Thus, we speculated that SENP1 down-regulation increases the degradation of HIF-1α. On the basis of the role of HIF-1α in cancer cells and our findings, we postulated that the SENP1 down-regulation in Daudi cells down-regulate HIF-1α protein expression, which then affected the transcription and post-transcriptional regulation of downstream factors including apoptosis related factors. This makes cancer cell unable to adapt to the hypoxic environment in the cancer resulting in apoptosis of cancer cells (Barliya et al., 2011; Du et al., 2011; El et al., 2011; Freise et al., 2011; Sapra et al., 2011; Liao et al., 2012).

Taken together, down-regulation of SENP1 expression in Daudi cells can reduce the stability of HIF-1α protein and induce the apoptosis of Daudi cells. Thus, gene therapy targeting SENP1 may become an adjunctive therapy of Burkitt lymphoma.

Acknowledgements

References

Bae SH, Jeonga JW, Park JA, et al (2004). Sumoylation increases HIF-1αstability and its transcriptional activity. *Biochem Biophys Res Commun*, **324**, 394-400.

Barliya T, Mandel M, Livnat T, et al (2011). Degradation of HIF-1alpha under hypoxia combined with induction of Hsp90 polyubiquitination in cancer cells by hypericin: a unique cancer therapy. *PLoS One*, **6**, e22849

Bawa-Khalfe T, Cheng J, Wang Z, Yeh ET (2007). Induction of the SUMO-specific proteasel transcription by the androgen receptor in prostate cancer cells. *J Biol Chem*, **282**, 37341-9.

Chavez JC, LaManna JC (2002). Activation of hypoxia-induced factor-1 in the rat cerebral cortex after transient global ischemia: potential role of insulin-like growth factor-1. *J Neurosci*, **2**, 8922-31.

Cheng J, Kang XL, Zhang S, et al (2007). SUMO-Specific protease l is essential for stabilization of HIFl alpha during hypoxia. *Cell*, **131**, 584-95.

Du J, Xu R, Hu Z, Tian Y, et al (2011). PI3K and ERK-induced Rac1 activation mediates hypoxia-induced HIF-1α expression in MCF-7 breast cancer cells. *PLoS One*, **6**, e25213.

El Guerrab A, Zegrour R, Nemlin CC et al (2011). Differential impact of EGFR-targeted therapies on hypoxia responses: implications for treatment sensitivity in triple-negative metastatic breast cancer. *PloS One*, **6**, e25080

Freise C, Ruehl M, Erben U, et al (2011). A hepatoprotective Lindera obtusiloba extract suppresses growth and attenuates insulin like growth factor-1 receptor signaling and NF-kappaB activity in human liver cancer cell lines. *BMC Complement Altern Med*, **11**, 39.

Jean M (2004). How cells endure low oxygen. *Science*, **303**, 1454-6.

Liao HY, Wang GP, Gu LJ, et al (2012). HIF-1α siRNA and cisplatin in combination suppresstumor growth in a nude mice model of esophageal squamous cell carcinoma. *Asian Pac J Cancer Prev*, **13**, 473-7.

Resar JR, Roguin A, Voner J, et al (2005). Hypoxia-inducible factor-1αpolymorphism and coronary collaterals in patients with is chemic heart disease. *Chest*, **128**, 787-91.

Sapra P, Kraft P, Pastorino F et al (2011). Potent and sustained inhibition of HIF-1α and downstream genes by a polyethyleneglycol-SN38 conjugate, EZN-2208, results in anti-angiogenic effects. *Angiogenesis*, **14**, 245-53

Shalizi A, Gaudilliere B, Yuan ZQ et al (2006). A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science*, **311**, 1012-7.

Shen ZX, Zhu XZ (2003). Malignant lymphoma. Beijing: People's Health Publishing House, 532-45.

Song T, Zhang X, Wang C, et al (2011). MiR-138 suppresses expression of hypoxia-inducible factor 1α (HIF-1α) in clear cell renal cell carcinoma 786-O cells. *Asian Pac J Cancer Prev*, **12**, 1307-11.

Wang HR, Gu CH, Zhong JH, et al (2006). Combined use of flow cytometry and G418 to screen the recombinant plasmid transfected cells. *J Diagn Concep Pract*, **5**, 52-5.

Yates KE, Korbel GA, Shtutman M, et al (2008). Repression of the SUMO-specific protease Senp1 induces p53-dependent premature senescence in normal human fibroblasts. *Aging Cell*, **7**, 609-21.

Yeh ET, Gong L, Kamitani T (2000). Ubiquitin-like proteins: new wines in new bottles. *Gene*, **248**, 1-14 .

Zhou W, Dosey TL, Biechele T, et al (2011). Assessment of hypoxia inducible factor levels in cancer cell lines upon hypoxic induction using a novel reporter construct. *PLoS One*, **6**, e27460.