European **Rev**iew for **Med**ical and **Pharmacol**ogical **Sci**ences | 2018; 22: 4551-4556

Expression of SIRT1 gene in human lung cancer lines enhances their sensitivity to the anticancer effects of cisplatin

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Abstract. – **OBJECTIVE:** Lung cancer is one of the lethal cancers and one of the major causes of cancer-related mortalities across the globe. SIRT1 gene has been reported to be involved in the progression and tumorigenesis of several types of cancers. However, the role of SIRT1 gene is in the progression of lung cancer is poorly understood. Against this backdrop, the present study was designed to investigate the expression of SIRT1 gene in different lung cancer cell lines. Moreover, the relation between the expression of this gene and the sensitivity of lung cancer cell lines to the anticancer effects of cisplatin was also investigated.

MATERIALS AND METHODS: Expression of SIRT1 gene was determined by quantitative RT-PCR. Protein expression was examined by Western blotting. Anti-proliferative activity was determined by MTT and colony formation assay. Apoptotic populations were determined by annexin V/IP staining and flow cytometry.

RESULTS: The results revealed that NCI-H125 showed lowest, NCI-H226 showed moderate, while as NCI-H358 exhibited the highest expression of SIRT1. The three differentially SIRT1 expressing cancer cell lines were subjected to cisplatin treatment. It was observed that cisplatin exhibited the highest anticancer activity against NCI-H125 (IC50, 1.25 μM) and lowest against NCI-H358 (IC50, 4.5 μM). Moreover, cisplatin leads to highest inhibition of colony formation and apoptosis in NCI-H125 and lowest against NCI-H358.

CONCLUSIONS: Expression of SIRT1 gene determines the sensitivity of lung cancer cells to anticancer effects of cisplatin. This work will pave for understanding the role of SIRT1 gene in cancer progression.

Key Words:
SIRT1, Cisplatin, Lung cancer, Apoptosis.

Introduction

Lung cancer is considered as one of the main causes of cancer-associated mortality across the globe[1]. Moreover, it is a major reason of cancer related deaths in China. The mechanisms of lung cancer and many other cancers are poorly understood. Several genes have been implicated in the progression of cancer in human[2]. Silent information regulator 2 (SIR2) is an anti-aging gene that was originally discovered in budding yeast, which encodes a protein with NAD^+ dependent histone deacetylase activity[3]. SIRT1 is one of seven homologs of SIR2 in mammals[4], which are involved in cell energy metabolism, proliferation, senescence, multiple inflammatory processes, neuroprotection, and tumorigenesis amongst others[5]. There are many controversies on the role of SIRT1 in tumors[6]. The controversy over whether SIRT1 serves as a tumor promoter or a tumor suppressor has not been completely resolved and the discussion will likely continue[6]. In the current study we attempted to investigate the role of SIRT1 gene in lung cancer progression. Firstly, we determined the expression of SIRT1 gene in five different human lung cancer cell lines (NCI-H125, NCI-H157, NCI-H226, NCI-H358 and NCI-H661) by Real-time quantitative RT-PCR. Out these three cancer cell lines NCI-H125, NCI-H226 and NCI-H358 were selected based on the SIRT1 gene expression. NCI-H125 exhibited lower expression, NCI-H226 exhibited moderate while as NCI-H358 exhibited the highest expression of SIRT1. We also confirmed the SIRT1 protein expression in all these three lung cancer cell lines by Western blotting. Secondly, we treated all the three cell lines to the

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known anticancer drug, cisplatin. It was observed that the expression of SIRT1 gene correlated well with the sensitivity of lung cancer cell lines to cisplatin. The cells with higher expression of SIRTI were less sensitive than cells with lower expression of SIRT1. The sensitivity of lung cancer cell lines to cisplatin was determined by cell viability, colony formation and apoptotic assays. We, therefore, propose that SIRT1 gene contributes to the tumorigenic potential of lung cancer cells and, in part, determines their sensitivity to the anticancer drug cisplatin *in vitro.*

Materials and Methods

Reagents, Cell Lines and Culture Conditions

Cisplatin, DAPI, RNase A triton X-100 dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fetal bovine serum (FBS), Roswell Park Memorial Institute 1640 (RPMI-1640) medium, L-glutamine, antibiotics were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Five human lung cancer cell lines (NCI-H125, NCI-H157, NCI-H226, NCI-H358 and NCI-H661) were purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO_2. All of the reagents were procured from Hyclone (Logan, UT, USA).

RNA Isolation, cDNA Synthesis and Quantitative RT-PCR Analysis

Total RNA of all the five-lung cancer cell line was isolated by RNeasy RNA isolation kit (Qiagen, Co., Ltd., Shanghai, China) as per the manufacturer's instructions. Thereafter, cDNA was synthesized with the help of RevertAid cDNA synthesis kit (Fermentas, Dublin, OH, USA) with manufacturer's protocol. To carry out the Real-time PCR, the cDNA was diluted 20 times and qRT-PCR was carried out in triplicates in ABI StepOne Real-time (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Fermentas, Dublin, OH, USA) and gene specific primers (Forward: 5'-GGTCGATGCTGCAATTCGCC-3' and Reverse: 5'-AACCACTGCTCGGTTCAATG-3'). The relative quantification method ($\Delta\Delta^{-CT}$) was used to determine quantitative variation between the replicates examined. The actin was used as positive control to normalize the data.

Western Blotting Analysis

After treatment with various concentrations of cisplatin, the lung cancer cells were collected and lysed in lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Nonidet P 40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail). From each protein sample, 20 µl aliquot was separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis SDS-PAGE gel. The gel was then transferred to nitrocellulose membranes, blocked with 5% bovine serum albumin (BSA) and probed with a primary antibody. This was followed by probing with the required secondary antibody. Finally, the signal was perceived with West-Save Up™ luminal-based enhanced chemiluminescence (ECL) reagent (ABFrontier, Seoul, Korea).

Anti-proliferative Activity Assay

MTT assay was used to determine the anti-proliferative activity of cisplatin against the human lung cancer cell lines as described previously[7]. Each of the three lung cancer cells in 100 µL culture medium were seed in a 96-well plate at a density of 3 × 10^3 cells/mL and kept at 37 °C in 5% CO_2 for a time period of 24 h. After 24 h, an additional 100 µL of complete medium with either no additions or different concentrations (0.62-10 µM) of cisplatin were added. Thereafter, the cells were incubated for 72 h. This was followed by the addition of 20 µL of MTT solution (5 mg/mL) and an incubation of 4 h. Afterwards, the medium was removed and 150 µL of dimethyl sulfoxide (DMSO) was added. The absorbance (OD) of each well was measured at 490 nm using a Tunable Mi-185 microplate Reader (EL-x 800, BioTek Instruments, Winooski, VT, USA).

Colony Assay

For colony formation assay, the lung cancer cells were seeded in a 12-well culture plates with approximately 500 cells per well and permitted to adhere for 12 h and administrated with 0, 1.5 and 3 µM concentrations of cisplatin. This was followed by incubation period of one week. The cells were then washed with phosphate buffered saline (PBS) and finally fixed with methanol for 30 min. Thereafter, the cells were stained with crystal violet (0.5%) solution.

Analysis of Apoptotic Cells by Flow Cytometry

The three-lung cancer cell lines (NCI-H358, NCI-H661, NCI-H125) at a density of 2×10^5 cells/well were platted in 6-well plates and administrated with 0, 1.5, and 3 μM concentration of cisplatin for 24. For estimation of apoptotic cell populations a FITC-Annexin V/PI apoptosis detection kit was used following the manufacturer's instructions (Biosea Biotechnology, Beijing, China) as described previously[8].

Statistical Analysis

All experiments were carried out in triplicates and the values were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way or two-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA). The values were considered significant at $^{*}p<0.01$, $^{**}p<0.001$ and $^{***}p<0.0001$.

Results

Expression of SIRT1 Gene in Human Lung Cancer cell Lines

In order to examine the transcript levels of SIRT1 gene, total RNA was isolated from six human lung cancer cell lines (NCI-H125, NCI-H157, NCI-H226, NCI-H358 and NCI-H661) and cDNA was synthesized. Thereafter, using gene specific primers, expression of SIRT1 gene was determined by quantitative RT-PCR (Figure 1). The results indicated that the five human lung cancer cell lines showed differential expression pattern of SIRT1 gene. The highest expression of SIRT1 gene was observed in NCI-H358 followed by NCI-H661. Comparatively moderate expression of SIRT1 gene was observed in NCI-H661 and lowest expression was observed in NCI-125. Based on the transcript levels of SIRT1 gene, three cell lines NCI-H358 (high expression), NCI-H226 (moderate expression) and NCI-H125 (lowest expression) were selected for further studies.

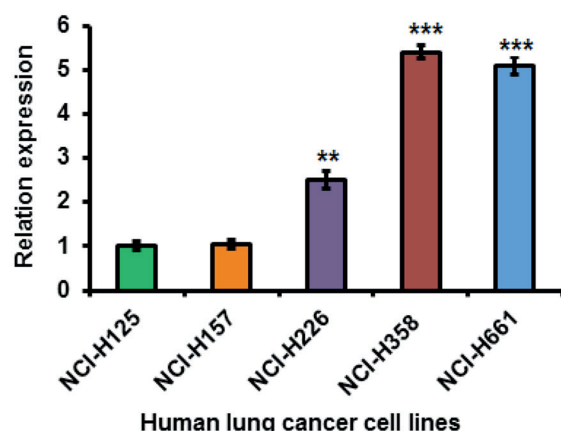

Figure 1. Expression of SIRT1 gene in human lung cancer cell lines as determined by quantitative RT-PCR. All experiments were done in three biological triplicates and expressed as mean ± SD. The values were considered significant at $^{*}p<0.01$, $^{**}p<0.001$ and $^{***}p<0.0001$.

Investigation of SIRT1 Protein Expression in Human lung Cancer Cell Lines

Three cell lines NCI-H358, NCI-H226, NCI-H125 selected based on the transcript levels of SIRT1 gene, were used for the determination of SIRT1 protein expression by Western blotting. The results of the study indicated that NCI-H358 has the highest SIRT1 protein expression followed by NCI-H226 and lowest expression was observed in NCI-125 (Figure 2a and 2b). The SIRT1 protein expression positively correlated with the

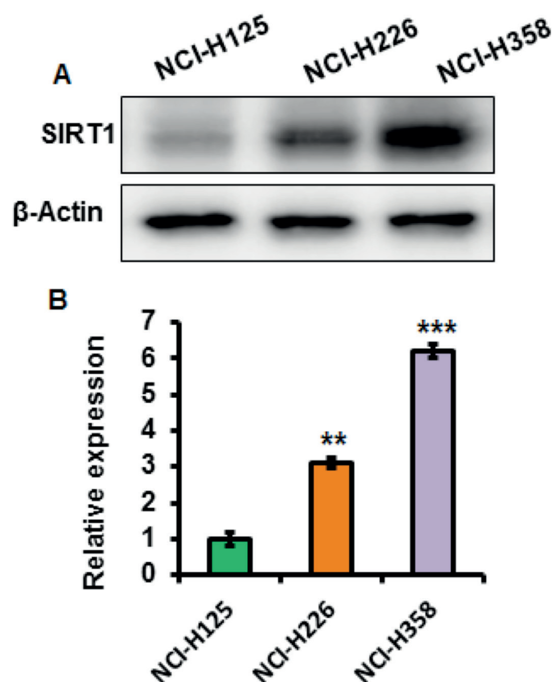

Figure 2. Western blot analysis showing ***(A)*** expression of SIRT1 protein ***(B)*** Densitometric quantification of SIRT1 expression in three human lung cancer cell lines. All expressions were carried out in three biological experiments and expressed as mean ± SD. The values were considered significant at $^{*}p<0.01$, $^{**}p<0.001$ and $^{***}p<0.0001$.

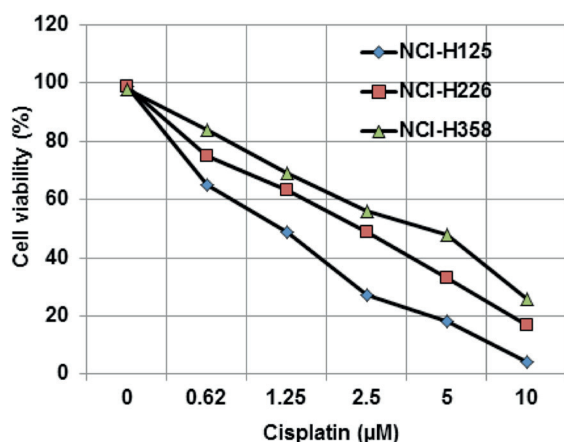

Figure 3. Anti-proliferative activity of cisplatin at indicated doses on the cell viability of three human lung cancer cell lines. All experiments were carried out in triplicates and expressed as mean ±SD.

transcript levels of SIRT1 gene in all the three human lung cancer cell lines.

Anti-proliferative Activity of Cisplatin against Differentially SIRT1 Expressing Human Lung Cancer Cell Lines

We evaluated anticancer effects of cisplatin on three selected human lung cancer cell lines (NCI-H358, NCI-H226, NCI-H125) exhibiting differential expression of SIRT1 gene. The results indicated that NCI-H125 with lowest expression of SIRT1 gene was more sensitive to the anticancer effects of cisplatin with an IC_{50} of 1.25 µM (Figure 3). Similarly, the human lung cancer line NCI-H358 highly expressing SIRT1 gene showed lowest highest sensitivity to cisplatin with an IC_{50} value of 4.5 µM while as the moderately SIRT1 expressing NCI-H226 cancer cell line exhibited moderate sensitivity to cisplatin with an IC_{50} of 2.5 µM. Therefore, the results indicate that expression of SIRT1 protein positively correlates with the sensitivity of lung cancer cells to cisplatin.

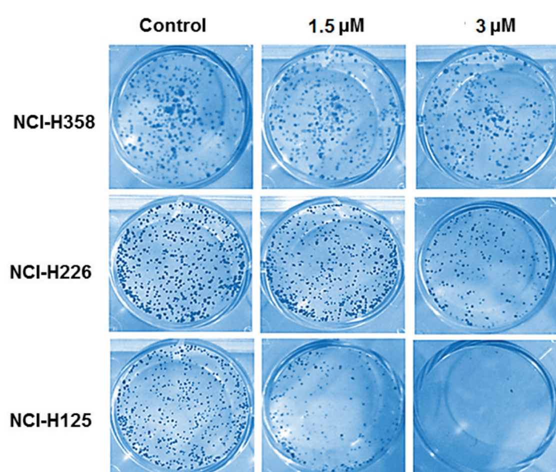

Figure 4. Effect of indicated doses of cisplatin on colony formation of differentially SIRT1 expressing human lung cancer cell lines. The experiments were carried out in triplicates.

Inhibition of Colony Formation in Human Lung Cancer Cell Lines Correlates with the Expression of SIRT1

To investigate the effect of cisplatin on the colony formation potential of the three human lung cancer cell lines differentially expressing SIRT1 gene, the cells were subjected to 0, 1.5 and 3 µM concentrations of cisplatin. The results indicate that cisplatin inhibited colony formation more in NCI-H125 followed by NCI-226 and NCI-358 (Figure 4). The results correlated well with the expression of SIRT1 gene in these lung cancer cell lines.

Apoptotic Cell Death in Human Lung Cancer cell Lines Correlates with the Expression of SIRT1

The correlation between the apoptotic cell death and the expression of SIRT1 gene was investigated by subjecting the differentially SIRT1 gene expressing human lung cancer cell lines to 0, 1.5 and 3 µM concentrations of cisplatin (Figure 5). The annexin V/IP staining followed by flow cytometry results revealed that cisplatin-induced apoptosis was higher in NCI-H125 lung cancer cell line followed by NCI-H226 and lowest in NCI-H358. The apoptosis results correlated well with the expression of SIRT1 expression both at gene and protein levels, thus confirming the role of SIRT1 gene in tumorigenesis of lung cancer.

Discussion

Lung cancer is one of the lethal cancers and is one of the major causes of cancer related mortalities across the globe. The treatment options for lung cancer have been associated with a number of side effects and the underlying mechanism of lung cancer is yet poorly understood[1,2]. Although the role of SIRT1 in cancer has been debated owing to the conflicting results of several studies, in the present investigation we attempted to explore the role of SIRT1 in lung cancer. To

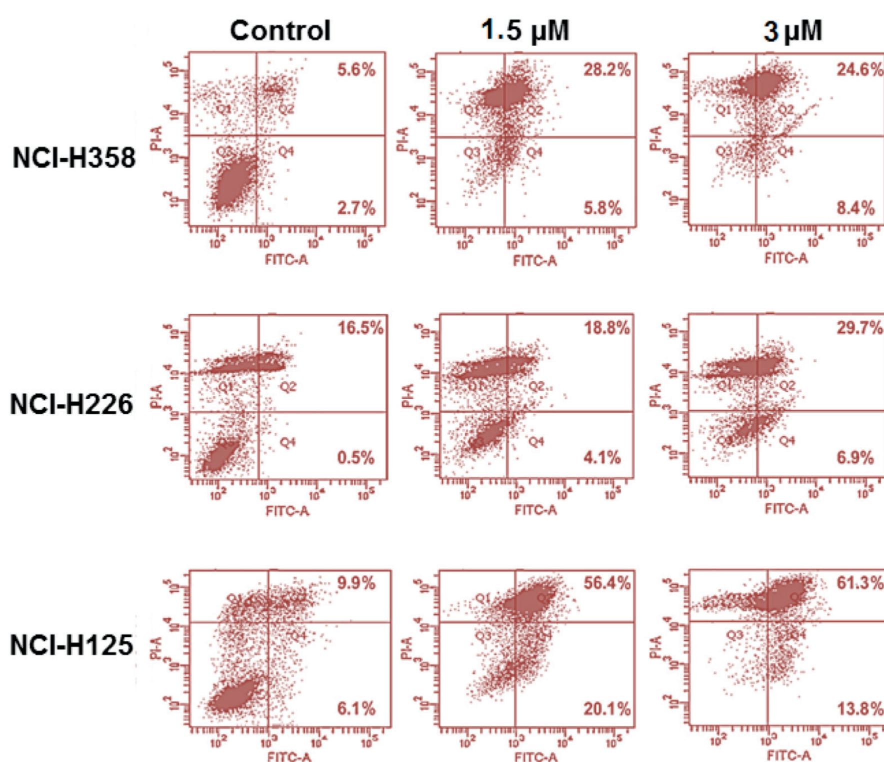

Figure 5. Effect of cisplatin on apoptosis induction in differentially SIRT1 expressing human lung cancer cell lines determined by flow cytometry. The experiments were carried out in three biological replicates.

the best of our knowledge, several earlier studies have investigated the correlation between SIRT1 expressions with prognosis of different cancers[9-11]; however, for the first time we report that SIRT1 gene expression contributes to the sensitivity of lung cancer cell lines to the anticancer effects of cisplatin. In the present work five different lung cancer cell lines (NCI-H125, NCI-H157, NCI-H226, NCI-H358 and NCI-H661) were examined for the expression of SIRT1 gene. Based on the quantitative RT-PCR analysis, three cell lines viz. NCI-H125 (with low expression of SIRT1) NCI-H226 (with moderate expression of SIRT1 gene) and NCI-H358 (with high expression of SIRT1 gene) were selected for further study. To confirm whether the transcript levels of SIRT1 gene translate into SIRT1 in the similar fashion, we determined the expression of SIRT1 protein by Western blotting. The results indicated that the results of quantitative RT-PCR positively correlated with the SIRT1 protein expression with highest expression in NCI-H358 and lowest in NCI-H25. Thereafter, the differentially SIRT1 protein expressing lung cancer cells were subjected to cisplatin treatment. Interestingly we observed that lung cancer cells with lower SIRT1 expression were more sensitive to anticancer effects of cisplatin as compared to cells with higher expression of SIRT1. Our results are in confirmation with several other researches wherein SIRT1 gene has been implicated in tumorigenesis and progression of several types of cancers[12-15]. Since the results of MTT assay are not enough to support our findings, we, therefore, evaluated the effect of cisplatin on the colony formation and apoptosis in all the three (NCI-H358, NCI-H226, NCI-H125) differentially SIRT1 expressing cells. Previous studies have demonstrated that cisplatin exerts its anticancer effects through inhibition of cell growth and induction of apoptosis[16]. The findings of the present are in confirmation with these previous studies. It was observed that cisplatin did inhibit colony formation and induced apoptosis in a done dependent manner. But more importantly, the highest inhibition of colony formation and apoptosis was observed in human lung cancer cell line NCI-125 and lowest in the NCI-H358, indicating that higher expression of SIRT1 is associated with lower sensitivity of the lung cancer cells to the anticancer effects of cisplatin and vice versa.

Conclusions

We showed that SIRT1 gene has an important role in lung cancer progression and its expression contributes to the sensitivity of lung cancer cells to anticancer drugs such as cisplatin. Therefore, this study will reinforce further in-depth research on SIRT1 gene and may eventually help in understanding the several underlying mechanisms of cancer progression and tumorigenesis in human.

Acknowledgments
This study was supported by Yongchuang Hospital of Chongqing Medical University Talents Funding Project (Project No: YJ201306 and Project No: 201503).

Conflict of interest
The authors declare no conflicts of interest.

References

1) Xia JJ, Zhao SF, Xiong LW, Chu TQ, Bai H, Yan B, Zhong RB, Shao MH, Han BH. Real-time PCR assay with high resolution melting for EGFR and BIM mutation of lung cancer. Eur Rev Med Pharmacol 2016; 20:2805-2811.
2) Shi WY, Liu KD, Xu SG, Zhang JT, Yu LL, Xu KQ, Zhang TF. Gene expression analysis of lung cancer. Eur Rev Med Pharmacol Sci 2014; 18: 217-218.
3) Fessel MR, Lira CB, Giorgio S, Ramos CH, Cano MI. Sir2-Related protein 1 from leishmaniaamazonensis is a glycosylated NAD+-dependent deacetylase. Parasitology 2011; 138: 1245-1258.
4) Imai S, Guarente L. Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. Trends Pharmacol Sci 2010; 31: 212-220.
5) Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. Biochem J 2007; 404: 1-5.
6) Song NY, Surh YJ. Janus-faced role of SIRT1 in tumorigenesis. Ann NY Acad Sci 2012; 1271: 10-19.
7) Ma L, Diao A. Marizomib a potent second generation proteasome inhibitor from natural origin. Anticancer Agents Med Chem 2015; 15: 298-306.
8) Kim SH, Choi KC. Anti-cancer effect and underlying mechanism (s) of kaempferol, a phytoestrogen, on the regulation of apoptosis in diverse cancer cell models. Toxicol Res 2013; 29: 229-234.
9) Lee H, Kim KR, Noh SJ, Park HS, Kwon KS, Park BH, Jung SH, Youn HJ, Lee BK, Chung MJ, Koh DH, Moon WS, Jang KY. Expression of DBC1 and SIRT1 is associated with poor prognosis for breast carcinoma. Hum Pathol 2011; 42: 204-213.
10) Suzuki K, Hayashi R, Ichikawa T, Imanishi S, Yamada T, Inomata M, Miwa T, Matsui S, Usui I, Urakaze M, Matsuya Y, Ogawa H, Sakurai H, Saiki I, Tobe K. SRT1720, a SIRT1 activator, promotes tumor cell migration, and lung metastasis of breast cancer in mice. Oncol Rep 2012; 27: 1726-1732.
11) Wu M, Wei W, Xiao X, Guo J, Xie X, Li L, Kong Y, Lv N, Jia W, Zhang Y, Xie X. Expression of SIRT1 is associated with lymph node metastasis and poor prognosis in both operable triple-negative and non-triple-negative breast cancer. Med Oncol 2012; 29: 3240-3249.
12) Saunders LR, Verdin E. Sirtuins: critical regulators at the crossroads between cancer and aging. Oncogene 2007; 26: 5489-5504.
13) Lim CS. Human SIRT1: a potential biomarker for tumorigenesis? Cell Biol Int 2007; 31: 636-637.
14) Wen YC, Wang DH, RayWhay CY, Luo J, Gu W, Baylin SB. Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses. Cell 2005; 123: 437-448.
15) Baylin SB, Ohm JE. Epigenetic gene silencing in cancer--a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 2006; 6: 107-116.
16) Azuma M, Tamatani T, Ashida Y, Takashima R, Harada K, Sato M. Cisplatin induces apoptosis in oral squamous carcinoma cells by the mitochondria-mediated but not the NF-kappaB-suppressed pathway. Oral Oncol 2003; 39: 282-289.