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Facilitation of liver cancer SMCC7721 cell aging by sirtuin 4 via inhibiting JAK2/STAT3 signal pathway

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Abstract. – OBJECTIVE: Liver cancer severely threatens public health. Molecular targeted treatment is the further of cancer treatment. The functional role of Sir-related enzymes 4 (sirtuin 4) in treating liver cancer still requires further investigation. This study aimed to elucidate the effect of sirtuin 4 on aging of SMCC7721 liver cancer cell line, to underlying molecular mechanism and potential application in clinics.

MATERIALS AND METHODS: Adriamycin-induced aging model was established on SMCC7721 liver cancer cell line. Sirtuin 4 over-expression or siRNA plasmid was transfected. Cell aging was measured by β-galactosidase approach. Aging-related proteins P53 and P16 were quantified in Western blot, which also examined activation of Janus kinase 2 (JAK2) signal pathway. CP-690550 was used to suppress JAK2 signal pathway for measuring aging status of SMCC7721 cells.

RESULTS: In aged SMCC7721 cells, sirtuin 4 was up-regulated, whilst P53 and P16 protein levels were elevated, in accompanied with JAK2/STAT3 signal pathway. Transfection of sirtuin 4 over-expression plasmid or siRNA increased or decreased sirtuin 4 expression. Adriamycin-induced aging was enhanced or suppressed, accompanied with inhibited or potentiated JAK2 signal pathway in sirtuin 4 up-regulation or down-regulation cells, respectively. The usage of JAK2 signal inhibitor, CP-690550, enhanced Adriamycin-induced cell aging.

CONCLUSIONS: Sirtuin 4 facilitates Adriamycin-induced aging of SMCC7721 liver cancer cells via inhibiting JAK2/STAT3 signal pathway, thus providing one novel anti-cancer strategy.

Key Words
Sirtuin 4, JAK2/STAT3, Liver cancer cell, Cell aging.

Introduction

Liver cancer is one important factor leading to death in digestive system due to its high incidence[1]. However, the mechanism of liver cancer is still unknown. A previous study[2] believed that liver cancer cell growth was accompanied by enhanced proliferation, plus decreased cell apoptosis or aging were important reasons. Molecular targeted treatment is the further direction for liver cancer treatment. Currently, the major approach treating liver cancer targets cell apoptosis, while a few studies[3,4] have been performed regarding cell aging. Cell aging is one dynamic process with decreased potency for cell growth, proliferation and differentiation with cell cycle progression and time elapse[5,6]. Both growth and activity of cancer cells are not decreased with elongated cell life, leading to over-proliferation and body imbalance for cancer occurrence[7,8]. A current work believed that Sir-related enzymes (sirtuin) family plays a critical role in cell aging[9,10]. Sirtuin family has highly conserved amino acid sequence and similar structures or functions. Certain functions, however, may also exist for specific sirtuin family member[11,12]. Overall speaking, the role of sirtuin needs to be further investigated. Sirtuin family has wide biological functions. For example, sirtuin 2 decreases cell proliferation velocity, while sirtuin 1 is closely correlated with metastasis of lung cancer[13,14]. These suggested the probable involvement of sirtuin 4 in occurrence of progression of liver cancer[15]. Therefore, this research utilized SMCC7721 liver cancer cell model, on which possible role of sirtuin 4 in SMCC7721 liver cancer cells, to provide evidence for choosing molecular treating targets in liver cancer.

Materials and Methods

Liver cancer cell model and reagent

This study utilized liver cancer cell model SMCC7211, which was purchased from Microbial Culture Preservation Center (ATCC, Manassas, VA, USA). Beta-galactosidase for detecting cell aging was purchased from Dingguo Technology (Beijing, China). Liposome transfection reagent

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was purchased from Hualan Biological Engineering Inc. (Xinxiang, China). Antibiotics, culture medium and fetal bovine serum (FBS) were purchased from Beyotime (Haimen, China). The antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SiRNA for sirtuin 4 (5'-GTCCA CAATG TGCTA CGTGC-3' and 5'-TGATC AACGA TACTT CGTT-3') and sirtuin 4 over-expression plasmid were purchased from Genepharma (Shanghai, China).

Cell culture and induced aging model of SMCC7721 cells

SMCC7721 liver cancer cells were resuscitated and re-suspended in normal Dulbecco's Modified Eagle Medium (DMEM) for culture[16]. Adriamycin (1 μl at 10 μg/μl) was added to induce cell aging for further culture and assays.

Liposome transfection method

Following previously reported results[17], liposome reagent was used to transfect siRNA sirtuin 4 or controlled siRNA, or sirtuin 4 over-expression plasmid were transfected into SMCC7721 liver cancer cells. In brief, SMCC7721 liver cancer cells were cultured at 90% density. 1 μl (1 μg/μl) siRNA of sirtuin 4 or controlled siRNA, or sirtuin 4 over-expression plasmid were re-suspended in liposome transfection reagent Lipo2000. Culture medium was changed after 48 h for further experiments.

Cell aging assay

β-galactosidase approach was used to test aging status of SMCC7721 liver cancer cells. Based on previous reports[18], liposome reagent was used to transfect siRNA sirtuin 4 or controlled siRNA, and sirtuin 4 over-expression plasmid into SMCC7721 liver cancer cells for cell aging assay. In brief, cells were cultured in 96-well plate. After washing, 1 μl staining buffer containing β-galactosidase was added. Staining was performed, followed by microscopic observation, recording and statistics.

Western blot

SMCC7721 liver cancer cells after transfecting sirtuin 4/siRNA sirtuin 4. The concentration of lysis buffer was measured by Western blot. In brief, 8-μg cell protein suspension was added into sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for transferring to the membrane, which was blocked (1:800), followed by development and fixation. Eventually, gel-imaging system was used for taking pictures for valuating sirtuin 4 and JAK2/JAKs expression level. Those expression levels of sirtuin 4 and JAK2/STAT3 were compared between all groups of SMCC7721 liver cancer cells[15].

Effects of interference or over-expression of Sirtuin 4 on SMCC7721 liver cancer cells

Sirtuin 4 or siRNA for sirtuin 4-transfected SMCC7721 cells were collected to quantify protein level using Western blot[15].

Statistical Analysis

All data were analyzed by SPSS 14.0 software (SPSS Inc. Chicago, IL, USA). Student *t*-test was used for comparison between all groups of SMCC7721 cells. Significance was defined when $p<0.05$. All experiments were repeated for at least three times.

Results

Adriamycin-induced SMCC7721 aging cells had elevated aging-related protein P53 and P16 expression, enhanced Sirtuin 4 expression and blockage of JAK2 signal pathway

As shown in Figure 1A, adriamycin-induced SMCC7721 liver cancer cell aging model had significantly elevated aging-related proteins P53 and P16, indicating that this study successfully established adriamycin-induced liver cancer cell aging model for further studies. As shown in Figure 1B, sirtuin 4 level was increased in Adriamycin-induced SMCC7721 liver cancer cell aging model, indicating that sirtuin 4 level might be related to Adriamycin-induced liver cancer cell aging. As shown in Figure 1C, JAK2/STAT3 signal pathway was suppressed in Adriamycin-induced SMCC7721 liver cancer cell aging model, indicating probable relationship between sirtuin 4 level and Adriamycin-induced SMCC7721 liver cancer cell aging.

Transfection of Sirtuin 4 over-expression plasmid or siRNA elevated or suppressed Sirtuin 4 expression level in Adriamycin-induced SMCC7721 liver cancer cell aging model, along with enhanced/inhibited cell aging, and suppressed/potentiated JAK2/STAT3 signal pathway

As shown in Figure 2A, transfection of sirtuin 4 over-expression plasmid or siRNA increased or

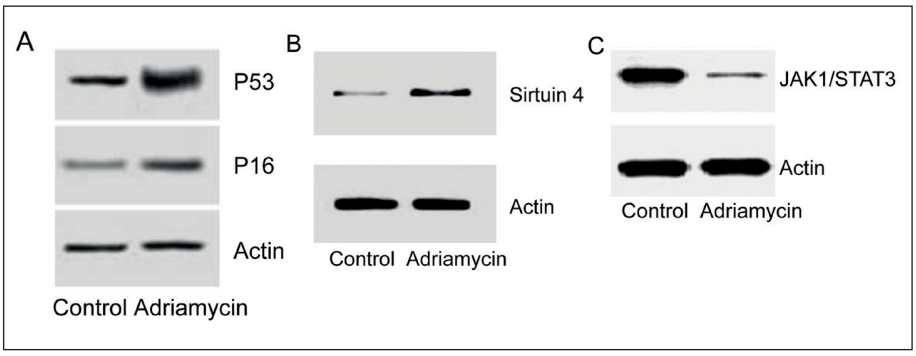

Figure 1. Adriamycin-induced SMCC7721 aging cells had elevated aging-related protein P53 and P16 expression, enhanced Sirtuin 4 expression and blockage of JAK2 signal pathway.

decreased sirtuin 4 expression level in SMCC7721 liver cancer cells, suggesting that this model can be used to investigate the effect of differential sirtuin 4 expression level on cell aging.

As shown in Figure 2B, in Adriamycin-induced SMCC7721 liver cancer cell aging model, transfection of sirtuin 4 over-expression plasmid or siRNA enhanced or inhibited aging of SMCC7721 liver cancer cells, indicating the involvement of sirtuin 4 in Adriamycin-induced SMCC7721 liver cancer cell aging. As shown in Figure 2C, in Adriamycin-induced SMCC7721 liver cancer aging model, transfection of sirtuin 4 over-expression plasmid or siRNA inhibited or enhanced JAK2/STAT3 signal pathway, indicating the involvement of JAK2/STAT3 signal pathway in Adriamycin-induced SMCC7721 liver cancer cell aging.

JAK2/STAT3 signal pathway inhibitor CP-690550 inhibited JAK2/STAT3 signal pathway and significantly enhanced Adriamycin-induced SMCC7721 liver cancer cell aging

As shown in Figure 3, the inhibition of JAK2/STAT3 pathway significantly enhanced Adriamycin-induced aging of SMCC7721 liver cancer cells.

Discussion

This study utilized SMCC7721 liver cancer cell as the model, from which the regulatory role and possible mechanism of sirtuin 4 on SMCC7721 liver cancer cell aging were investigated from both molecules and proteins. Results showed that transfection of sirtuin 4 enhanced expression level of aging molecules in human liver cancer cell SMCC7721, leading aging of SMCC7721 liver cancer cells, as consistent with previous studies, supporting the involvement of sirtuin 4 in cell growth and aging[19,20]. The molecular mechanism of sirtuin 4 in regulating growth and aging of liver cancer cell is still unknown[3]. Sirtuin 2 probably inhibits liver cancer growth, whilst sirtuin 1 is correlated with tumor metastasis[21,22]. These suggested that sirtuin 4 probably participated in occurrence and progression of liver cancer[23-25]. A previous study[26] showed that JAK2/STAT3 protein might be cell aging inhibition protein. In the present work whether JAK2/STAT3 is under the regulation of sirtuin 4 for further mediation of growth, and aging of SMCC7721 liver cancer cells is still unclear[27,28]. We showed that transfection of sirtuin 4 decreased JAK2/STAT3 level. After JAK2/STAT3 down-regulation by Sirtuin 4 transfection, aging rate of SMCC7721 liver can-

Figure 2. Transfection of Sirtuin 4 over-expression plasmid or siRNA elevated or suppressed Sirtuin 4 expression level in Adriamycin-induced SMCC7721 liver cancer cell aging model, along with enhanced/inhibited cell aging, and suppressed/potentiated JAK2/STAT3 signal pathway.

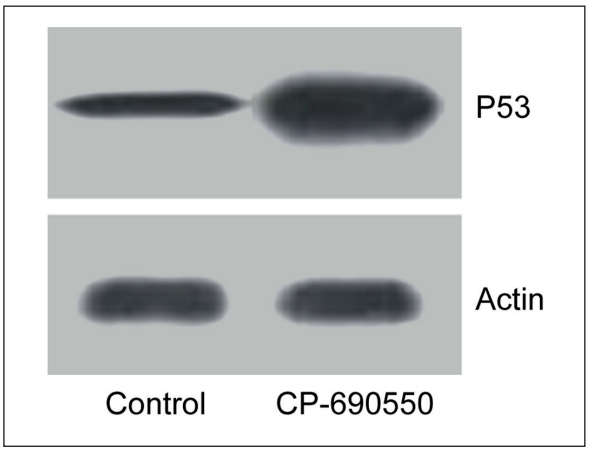

Figure 3. JAK2/STAT3 signal pathway inhibitor CP-690550 inhibited JAK2/STAT3 signal pathway and significantly enhanced Adriamycin-induced SMCC7721 liver cancer cell aging.

cer cell increased, whilst the inhibitor of JAK2/STAT3 signal pathway enhanced Adriamycin-induced aging of SMCC7721 liver cancer cells. As one recognized cell aging inhibitor, JAK2/STAT3 has no known effects on tumor occurrence or progression. However, by over-expression or interference of sirtuin 4 level, JAK2/STAT3 level was changed, indicating the important role of JAK2/STAT3 in Adriamycin-induced SMCC7721 liver cancer cell aging. In this study, we used three different results to demonstrate the role of sirtuin 4 and JAK2/STAT3 protein in Adriamycin-induced SMCC7721 liver cancer cell aging. Firstly, data showed elevated sirtuin 4 level in Adriamycin-induced SMCC7721 liver cancer aging model, plus over-expression of aging-related proteins P53 and P16, and inhibited JAK2/STAT3 signal pathway. Secondly, transfection of sirtuin 4 over-expression plasmid or siRNA could elevate or decrease sirtuin 4 expression in SMCC7721 liver cancer cells, enhanced/inhibited Adriamycin-induced SMCC7721 liver cancer cell aging, and blocked/potentiated JAK2 signal pathway. Thirdly, CP-690550 inhibited JAK2 and enhanced Adriamycin-induced SMCC7721 liver cancer cell aging. These results showed the critical role of sirtuin 4 and JAK2/STAT3 proteins in Adriamycin-induced SMCC7721 liver cancer cell aging. Targeting sirtuin 4 and JAK2/STAT3 protein might be novel strategy for molecular targeted treatment for liver cancer[26]. Currently, JAK2/STAT3 can inhibit aging of other tumor cells[21,23-28]. These results indicated that sirtuin 4 could induce SMCC7721 liver cancer cell aging via suppressing JAK2/STAT3 induction. This study has certain weakness. Firstly, we did not examine tumor tissues or adjacent tissues of liver cancer patients, thus we did not reveal sirtuin 4 or JAK2/STAT3 protein level in tumor or adjacent tissues from protein level, nor we did the investigation of the correlation between sirtuin 4 or JAK2/STAT3 expression level and liver cancer cell aging or liver cancer from a clinical perspective. Secondly, we did not acquire liver cancer tissues in those patients who received surgical resection, thus investigating the correlation between sirtuin 4 or JAK2/STAT3 expression level and liver cancer cell aging or liver cancer from clinical perspective. Thirdly, this study lacked the animal model for liver cancer. The usage of sirtuin 4 in animal experiment thus may reveal efficiency of sirtuin 4 targeted liver cancer from the animal level.

Conclusions

We showed that sirtuin 4 could facilitate Adriamycin-induced SMCC7721 liver cancer cell aging via suppressing JAK2/STAT3 signal pathway. This study provides possibly novel strategy against liver cancer, indicating that JAK2/STAT3 might be possible treating target for liver cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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