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Establishment of a Livin-gene silencing system and its effect on the sensitivity of Cal 27 with regard to 5-fluorouracil

Zixiao Huang[1,4], Ziwei Cui[1], Ruoshan Qin[1], Xuanxuan Yao[1], Hongli Zhou[1], Ru Guo[1], Xiaodong Qin[2,*], Xiangyi He[1,3,*]

[1]School of Dentistry, Lanzhou University, Lanzhou, 730000, P. R. China;

[2]Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Sciences, Lanzhou, Gansu, 730000, P. R. China;

[3]Key Laboratory of Functional Genomic and Molecular Diagnosis of Gansu Province, Lanzhou, 730030, P. R. China.

[4]Xiangyang Stomatological Hospital; Affiliated Stomatological Hospital of Hubei University of Arts and Science, Xiangyang, 441000, P. R. China.

***Co-corresponding authors:**

Dr. Xiaodong Qin, Lanzhou Veterinary Research Institute, No. 1 Yanchangbu Lu, Lanzhou, 730000, Gansu Province, P. R. China. Cell phone: +8618119463675; E-mail: qinxiaodong@caas.cn

Prof. Xiangyi He, School of Dentistry, Lanzhou University, No. 199 Donggang Xi Lu, Lanzhou, 730000, Gansu Province, P. R. China. Cell phone: +8613038730686; Fax: +86(931)8915051; E-mail: hexy@lzu.edu.cn

Abstract

Objective: This study aims at downregulating the expression of Livin through shRNA and exploring the sensitivity of Cal 27 to 5-fluorouracil (5-FU), so as to provide some help in resolving the chemotherapy resistance of oral cancer.

Methods: The Livin-targeting shRNA sequence was designed to construct a lentivirus and infect Cal 27 to obtain a stable Livin-silencing cell strain. After being treated with 20 μM, 40 μM and 60 μM of 5-fluorouracil for 24 h, cell apoptosis and cell viability were measured by flow cytometry and MTT assay, respectively.

Results: A shRNA-based lentivirus targeting Livin was successfully designed and constructed. After the silencing of Livin, the sensitivity and cell apoptosis of Cal 27 to 5-FU were dramatically elevated, and cell viability was significantly reduced (P<0.05). Moreover, the inhibition of Caspase 3 was observed.

Conclusion: The expression level of Livin was downregulated in Cal 27 after shRNA transfection, which increased the sensitivity of Cal to 5-FU. The underlying mechanism is the blockage of the activation of Caspase 3. Therefore, Livin may serve as a promising target for oral cancer treatment.

Keywords: Mouth Neoplasms; Inhibitor of Apoptosis Proteins; Drug Therapy; Caspase 3; Fluorouracil; Gene Silencing

Introduction

Oral cancer is ranked as the tenth most common cancer in humans, as its incidence reaches about 3.8% [1]. Every year, 300,000 to 700,000 patients worldwide are diagnosed with oral cancer, and its 5-year survival rate is merely 50% [2-4]. To date, the main treatment approach has been surgery combined with chemotherapy or radiotherapy, while the resistance or tolerance of cancer cells to chemotherapeutic drugs hinders our efficacy and prognosis.

Studies have revealed the causes of drug resistance, including unique tumor microenvironment, drug efflux, enhanced DNA repair, and overexpression of anti-apoptotic factors [5-7]. Among them, the IAPs (Inhibitor of Apoptosis Proteins), which are composed of XIAP, c-IAP1, c-IAP2, NAIP, ILP2, Survivin and Livin, are known to have anti-apoptotic functions. Livin is the most recently identified apoptosis inhibitor factor in IAP. Normally, Livin is expressed exclusively in terminally differentiated cells in adults, but substantial evidence suggests high expression of Livin in cancer tissues such as malignant melanoma, head and neck squamous cell carcinoma, lung adenocarcinoma cells and renal cell carcinoma [8-14]. Meanwhile, its aberrant expression may cause tumor cells to become insensitive or resistant to chemotherapeutic agents [15-18]. There is a lack of studies between Livin and the sensitivity of oral cancer cells to chemotherapy. Therefore, the aims of our study are to explore the chemotherapeutic sensitivity of oral cancer cells to the chemotherapeutics drug, 5-fluorouracil, after downregulating the expression of Livin, and to reveal its related underlying mechanisms.

Materials and methods

Cell thawing and culture. Human oral epithelial cells (HOECs) and periodontal ligament cells (PDLCs) were used in accordance with the ethical standards established in the Declaration of Helsinki. HOECs and PDLCs were stored, thawed and cultured as previously described [19-21]. HOECs were cultured in EpiGRO™ Human Epidermal Keratinocyte Complete Media Kit (Merck Millipore, Germany), and the medium for PDLCs and Cal 27 was α-MEM (Gibco, US) and DMEM (Gibco, US), respectively, and 10% (v/v) was supplemented with fetal bovine serum (FBS, Gibco, USA). These cells were thawed and then cultured in a T25 flask; as soon as 70% coverage was achieved, the cells were digested with 0.25% trypsin and passaged at a ratio of 1:3.

Construction of recombinant plasmid. Primers for shRNA targeting Livin were designed and are shown in Table 1. The vector plasmid pLKO.1 (#24150, Addgene) was digested with AgeI (#R0552S, New England Biolabs) and EcoRI (#R0101S, New England Biolabs) at

37℃ for 2 h. The primers were then ligated into digested pLKO.1 with T4 (#M0202S, New England Biolabs) at 4℃ for 12 h. 5 μL of the ligated product was added to 50 μL of competent cells and the following transformations were performed. Finally, plasmids were extracted and sequenced, and these recombinant plasmids were named pLV-shLivin.

Table 1. Primers for shRNA and RT-qPCR

Name	Sequence
shLivin-F	CCGGGCAGGAGGAGAGGACGTGCAACTCGAGTTGCACGTCCTCTCCTCCTGCTTTTTG
shLivin-R	AATTCAAAAAGCAGGAGGAGAGGACGTGCAACTCGAGTTGCACGTCCTCTCCTCCTGC
GAPDH-F	GGAGCGAGATCCCTCCAAAAT
GAPDH-R	GGCTGTTGTCATACTTCTCATGG
Livin-F	GCTCTGAGGAGTTGCGTCTG
Livin-R	CACACTGTGGACAAAGTCTCTT

Construction of lentivirus. The plasmids of pMD2.G and psPAX2 were mixed with pLV-shLivin or pLKO.1, respectively, for 15 min at room temperature according to the protocol (Attractene Transfection Reagent, QIAGEN), and then the mixture was added to 293T cells of 80% confluence. After being cultured for 48 h, the medium was collected separately and centrifuged at 1250 rpm for 5 min and stored in 4℃ for subsequent analysis.

Effects of lentivirus on Cal 27 cells. Cal 27 cells were seeded in a T25 flask, and the culture was refreshed when confluence reached 70%, followed by the addition of 1 mL of pLV-shLivin lentivirus and pLKO.1 lentivirus, respectively. After suspension of Cal 27 cells in the normal group, the medium for normal and affected cells was refreshed after 24 h and 10 μL of hygromycin was added to screen out successfully affected cells.

Relative expression of livin by RT-qPCR assay. Total RNA from HOEC, PDLC, Cal 27 and affected Cal 27 cells was extracted according to the protocol of RNeasy Mini Kit (Qiagen, Germany), followed by cDNA synthesized under guidelines of Maxima Minus cDNA

Synthesis Master Mix (Thermo Scientific, US) and primers, as listed in Table 1. Finally, RT-qPCR amplification was conducted according to Thermo Scientific DyNAmo Flash SYBR Green qPCR Kit. The relative expression of *livin* was determined by the $2^{-\Delta\Delta Ct}$ method and normalized to the relative expression of GAPDH. Each test was conducted three times.

Western blot analysis. Cal 27 cells of pLKO.1 and KD were harvested when 70% confluence was reached, and total protein was extracted separately according to the RIPA protocol. Subsequently, 4×loading buffer was added to each sample and heated to 95℃ for 10 min. Each 5 μL protein sample was added to SDS-PAGE and transferred to a PVDF membrane, which was then blocked with 5% skimmed milk solution for 4 h. The trimmed PVDF membranes were incubated at 4℃ for 12 h with the primary antibody, which was the Livin polyclonal antibody, and then incubated at room temperature for 1 h with the corresponding secondary antibody. The membranes were visualized and analyzed after treatment with Acrylamide Solutions under an Amersham lmager 600 system (General Electric Company, USA). Each sample was tested three times.

Apoptosis assay by flow cytometry. pLKO.1 and KD were harvested and seeded in a 96-well flask at 5×10^5 cells per well and cultured overnight and treated with 5-FU at concentrations of 0 μM, 20 μM, 40 μM and 60 μM, respectively. After being cultured for 24 h, cells were harvested and 50 to 100,000 of them were centrifuged at 1000 g for 5 min. Finally, following the protocol (C1062M, Beyotime, China), the cell pellet was resuspended in 195 μM Annexin V-FITC binding buffer and mixed with 5 μl Annexin V-FITC and 10 μl propidium iodide. Apoptosis analysis was conducted by incubation at room temperature for 15 min in a guava easyCyte system (Merck Millipore, Germany). Each sample was tested three times.

MTT assay for cell proliferation. Similarly, pLKO.1 and KD were harvested and seeded in a 96-well flask at 5×10^3 cells per well and cultured overnight. In addition, cells were treated with 0 μM, 20 μM, 40 μM and 60 μM 5-FU, respectively, and cultured for 24 h. Cells were incubated with 10 μL MTT solution for 4 h and with 100 μL SDS-HCl solution for 4 h according to the experimental protocol (V13154, Thermo Scientific, USA). Then, the OD values were read under SpectraMax Plus 384 system (Molecular Devices, USA). Each sample was tested three times.

Statistical analysis. Data were collected and presented as mean ± SD (n = 3). Calculations were performed using GraphPad Prism (version 7.04) and statistical differences were examined using analysis and *t*-tests.

Results

Relative expression of livin between Cal 27 and normal oral cells. The relative expression of *livin* was tested by RT-qPCR, and the results revealed significantly high levels of mRNA for *livin* in Cal 27 cells and low expression in HOECs and PDLCs. Comparisons of *livin* expression between Cal 27 and HOECs (1.01 vs. 0.00079, $P < 0.001$, Figure 1A) or between Cal 27 and PDLCs (1.01 vs. 0.1345, $P < 0.001$, Figure 1A) all exhibited statistically significant differences. Meanwhile, in cells originating from normal oral tissues, HOECs and PDLCs showed parallel expression of *livin* ($P > 0.05$, Figure 1A).

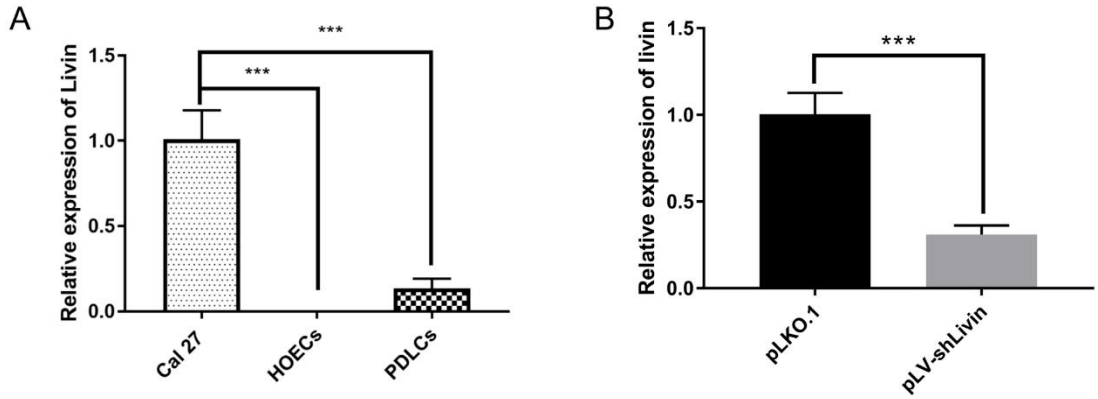

Figure 1. (A) Relative expression of *livin* in HOECs, PDLCs and Cal 27 as detected by RT-qPCR (***$P < 0.001$); (B) RT-qPCR analysis of the relative expression of *livin* in Cal 27 cells before and after gene silencing (***$P < 0.001$).

Knockdown of livin by shRNA in Cal 27 cell. The lentivirus-mediated pLKO.1 plasmid or pLV-shLivin plasmid affected Cal 27 cells separately and were screened by hygromycin. RT-qPCR and Western blot examined the expression of *livin* after shRNA knockdown. Compared to pLKO.1, *livin* in the pLV-shLivin group was obviously downregulated by about 70% (1.005 ± 0.07038 vs. 0.3093 ± 0.03001, $P < 0.05$, Figure 1B). Similarly, Western blot signified a downward trend in the protein level of Livin in the knockdown group (Figure 2).

Meanwhile, the Caspase 3 band was denser in the knockdown group than in the counterpart group, and gray value analysis showed a significant difference in Caspase 3 (P<0.05) (data not shown). All these represented the successful expression of shRNA and silencing effect of *livin* and the accompanying influence on Caspase 3.

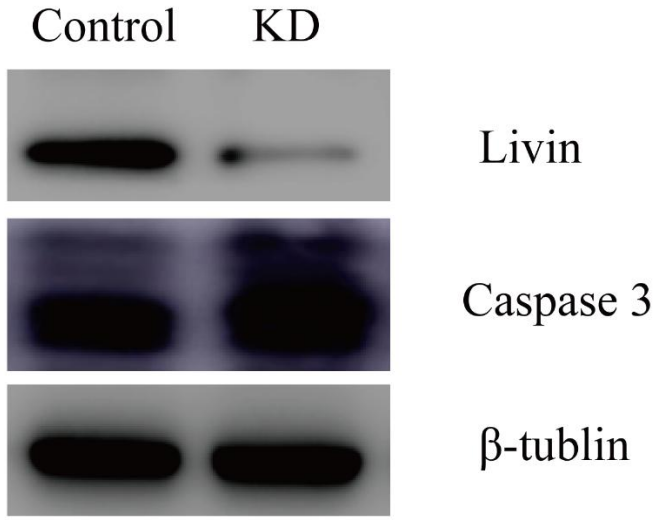

Figure 2. Western blot analysis of Livin and Caspase 3 after gene silencing. Control, Cal 27 in the pLKO.1 group; KD, Cal 27 in the pLV-shLivin group (***P < 0.001).

Sensitivity changes of Cal 27 to 5-fluorouracil after gene silencing. Altered sensitivity was measured by apoptosis assay and cell proliferation assay after treatment with 5-fluorouracil at different concentrations of 0 μM, 2 0μM, 40 μM and 60 μM. Flow cytometry results showed that the apoptosis rate in both the pLV-shLivin group and pLKO.1 group exhibited an increasing trend as the concentration of 5-FU increased. Moreover, apoptosis was much higher in the pLV-shLivin group compared to the pLKO.1 group (20 μM: 66.3% vs. 34.85%; 40 μM: 85.05% vs. 62.71%; 60 μM: 90.62% vs. 72.35%, P < 0.01) (Figure 3). MTT demonstrated cell viability by cell proliferation rate, which declined with increasing 5-FU concentration. For the gene knockdown group, Cal 27 descended faster than in the pLKO.1 group (20 μM: 84% vs. 73.33%; 40 μM: 56.87% vs. 39.4%; 60 μM: 28.83 vs. 14.53, P < 0.05) (Figure 4). The data above summarizes the upregulated sensitivity of Cal 27 to 5-FU after shRNA infection.

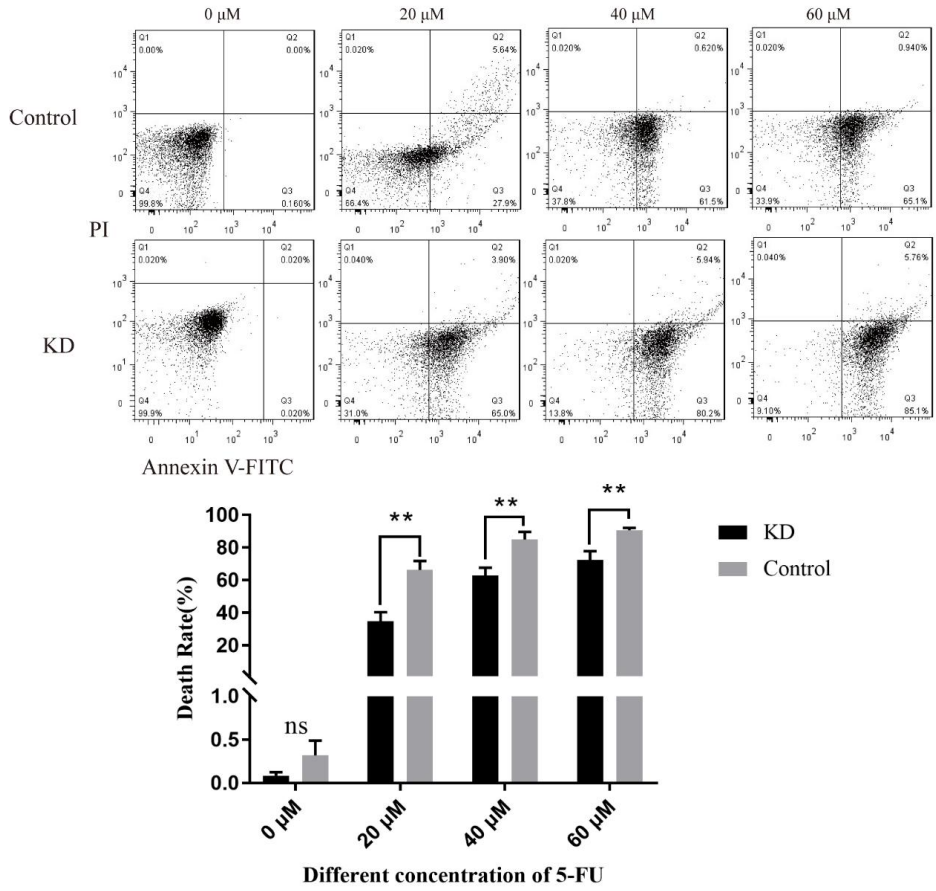

Figure 3. Flow cytometry assay of the apoptosis rate on Cal 27 cells treated with 5-FU. Control, Cal 27 in the pLKO.1 group; KD, Cal 27 in the pLV-shLivin group (**$P < 0.01$; ns, not significant).

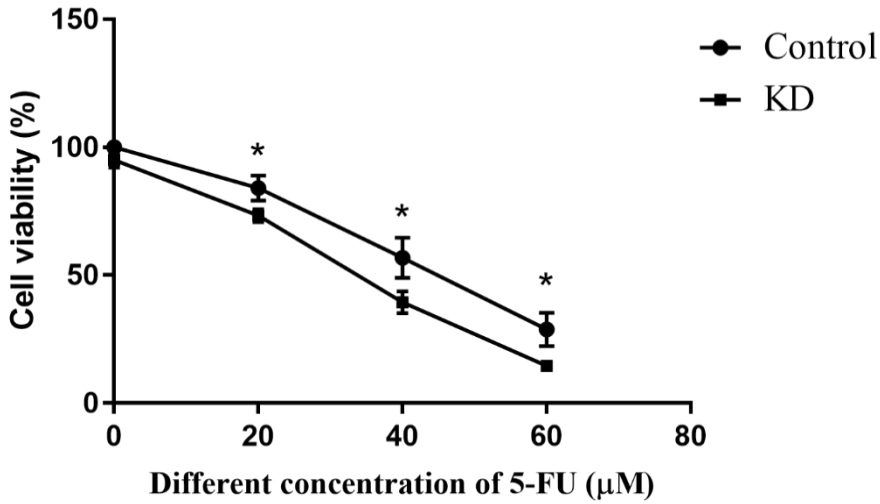

Figure 4. MTT assay of cell viability on Cal 27 cells treated with 5-FU. Control, Cal 27 in the pLKO.1 group; KD, Cal 27 in the pLV-shLivin group (*$P < 0.05$).

Discussion

Oral cancer is an aggressive malignant tumor that causes dysfunction of oropharyngeal tissues, aesthetic sequelae and reduces the quality of life of patients. The poor prognosis of oral cancer is mainly attributed to its delayed detection and histological features. According to the report of the FDI *Oral Health Atlas*, oral cancer occurs mostly in middle-aged men whose average age at the time of diagnosis is about 60 years. Histopathological classification of oral cancer usually manifests as squamous cell carcinoma, which can usually confer chemoresistance or radiation therapy on cancer cells.

In clinical practice, multidisciplinary therapy for patients with malignant oral cancer often involves surgical resection and post-operative radiotherapy or chemotherapy, whose final outcome is always beset by sensitivity to radiation or chemotherapeutic drugs, such as cisplatin, 5- fluorouracil (5-FU) and docetaxel. The histological classification of oral cancer subtypes not only impedes the prognosis, but also many other aspects of the matter, and studies have shown drug efflux, drug inactivation, DNA repair enhancement, anti-apoptotic element overexpression, etc. Inhibitors of Apoptosis Proteins (IAPs) are a family of proteins that can promote cell survival, regulate cellular differentiation, immunize and prevent apoptosis activity, especially in cancer cells. Human IAPs consist of 8 members, namely NAIP, cIAP1, cIAP2, XIAP, Survivin, BRUCE, Livin and ILP2, and all these proteins share a highly evolutionary conserved motif called baculoviral IAP repeat (BIR), whose interaction with the Caspase family is pivotal through its anti-apoptotic effect [6,22] . Structurally, the C-terminal Really Interesting New Gene (RING) domain has E3 ligase activity.

Livin, encoded by *BIRC7*, is the latest homologue discovered in the IAP and consists of the BIR domain and RING domain. There is increasing evidence showing that deregulated Livin is overexpressed in various cancers or carcinomas, such as acute myelocytic leukemia, osteosarcoma, lung adenocarcinoma, adrenocortical tumors, malignant melanoma and malignant parotid gland, and that this aberrant expression potently correlates with poor prognosis and low response to antitumor therapy. Meanwhile, Livin is barely expressed in highly differentiated human cells. In our study, RT-qPCR revealed the trend of *livin* expression between normal oral cells, HOECs and PDLCs, and oral cancer cells – Cal 27.

Correspondingly, *livin* was distinctly higher in Cal 27 than in normal oral cells ($P < 0.05$). Besides, *livin* was barely found in HOECs and PDLCs, and there was no statistical difference within these two normal cells. In addition, our early pilot study on immunohistochemistry and RT-qPCR assay of Livin in samples of buccal mucosa carcinoma and malignant parotid gland tumor accordingly displayed high levels of Livin (data not shown). The data for Livin in malignant lesions also indicate attenuated results of chemotherapy or radiotherapy.

Consequently, the preference for Livin expression in oral cancer prompted us to figure out the effect of downregulation of Livin on the response of Cal 27 to chemotherapy agents, such as 5-FU. The shRNA targeting *livin* was predesigned and subsequently constructed as a lentivirus to achieve the desired affection and expression efficiency. Moreover, the knockdown effect of Livin on the screened-out Cal 27 cells was tested through RT-qPCR and Western blot, manifesting a reduced expression level and obvious fading of the bands in the pLV-shLivin group ($P < 0.05$). The results of flow cytometry assay showed that the rate of 5-FU-induced apoptosis varied with increasing concentration in response to increasing dose, and the cell death rate in the pLV-shLivin group was higher than that in the same 5-FU concentration ($P < 0.05$). Similarly, drops of Cal 27 cells silenced with Livin in MTT assay became steeper with 5-FU that inhibited the proliferation of Cal 27 cells.

The above results imply an increased sensitivity of Cal 27 cells to 5-FU after Livin gene silencing, mainly embodied by apoptosis rate. In most cases, the initiation and cascade reaction of the apoptosis pathway relates to the Caspase family. Basically, apoptosis-related Caspase proteins can be categorized into two subsets. The initiators, such as Caspase 8/9/10, can be recruited into cell membrane death receptor complexes, (e.g., TNFR1, Fas and TRAIL-R1/2) to dimerize and self-cleave into an active state. The other group is the executioner, such as Caspase 3/6/7, which functions in the active state by cleaving from the pro-Caspase zymogens that depend on the previous activation of initiator Caspases [23-25] . Finally, the catalytically executioner caspases can cause massive protein proteolysis, especially for Caspase 3, including poly (ADP-ribose) polymerase (PARP) and Lamin A, resulting in nuclear DNA instability and cellular metabolic disintegration. The entire cell death pathway acts under surveillance and can be antagonized by Livin, typically in

numerous cancer cells. Meanwhile, Livin may interact with couples of Caspase subunits, such as Caspase 3/7/9, and is highly contextualized to the cell line. Gary M. Kasof [26] reported in HeLa cells that Livin could bind to Caspase 3/7, but had no obvious effect on enzyme activity. Feng Jin [27] used temozolomide (TMZ) as stimulus, which triggered the inhibitory effect on Livin in TJ905 glioma cells and increased the expression of Caspase 3/7/9. Deyong Yang [28] knocked down Livin in T24 human bladder cancer cell lines and prompted the expression of Caspase 3/9, whereas Caspase 7 remained unchanged. Studies have shown that overexpression of Livin may inhibit the activation and function of Caspase 3 and that this inhibition can be alleviated by downregulating Livin [29,30] . Therefore, our study mainly focused on its relation to Caspase 3. After lentivirus-mediated downregulation of Livin by shRNA, the Caspase 3 band of pLV-shLivin in Western blot appeared thicker, implying a higher expression level of Caspase 3 in Cal 27 cells that is parallel to that of Livin. Cal 27 is a typical oral cancer cell line that possesses uncontrolled reproduction, constant invasion and a unique metabolism that results in cell proliferation overwhelmed rather than cell death. Untreated with any stimuli, it also expresses Caspase 3, but without obvious apoptosis, probably through an interaction between Caspase 3 and the BIR structural domain of Livin, leading to an attenuated cell death pathway. Upon hydrolysis of Livin's mRNA by shRNA, inhibition of Caspase 3 was suspended and resistance to 5-FU was alleviated.

In conclusion, Livin is highly expressed in oral cancer cells Cal 27 and may inhibit the activation of Caspase 3. The gene silencing effect of Livin consequently revealed an increased sensitivity of Cal 27 induced by 5-FU. Correspondingly, Livin may serve as a promising target for oral cancer treatment. Therefore, our further research may introduce additional chemotherapeutic drugs, such as platinum compounds or docetaxel, and consider more cell lines, such as SCC 9 or SCC 25.

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