·Basic Research·

A G-quadruplex ligand SYUIQ-5 induces autophagy by inhibiting the Akt-FOXO3a pathway in nasopharyngeal cancer cells

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[Abstract] Background and Objective: As a new cryptolepine derivative containing indole and quinoline structures, SYUIQ-5 has been reported to induce and stabilize G-quadruplex, inhibit c-myc promoter and telomerase activity. This study was to investigate autophagy induced by a G-quadruplex ligand SYUIQ-5 and its mechanisms in nasopharyngeal cancer cells. Methods: The protein levels of microtubule-associated protein 1 light chain 3 (LC3), Akt, p-Akt, autophagy-related genes BNIP3 (adenocarcinoma E1B19KD interacting protein 3) and Beclin1 were determined by Western blot in nasopharyngeal cancer cell lines CNE1, CNE2 and HONE1. The mRNA levels of LC3 and BNIP3 was detected using reverse transcription polymerase chain reaction (RT-PCR). RNA interference was used to block the expression of BNIP3 and the effect of BNIP3 was evaluated in SYUIQ-5-induced autophagy. The localization of FOXO3a was observed using confocal immunofluorescence. Results: The protein and mRNA levels of LC3 in CNE1, CNE2 and HONE1 were up-regulated in a dose-dependent manner after being treated with 0.25-2 μ g/mL SYUIQ-5 for 48 h. Incubation of CNE2 cells with SYUIQ-5 markedly inhibited the phosphorylation of Akt, but did not statistically change the total Akt level. After incubation with 3μ g/mL SYUIQ-5 for 24 h, nuclear translocation of FOXO3a was observed under confocal immunofluorescence in CNE2 cells. Autophagy-related gene BNIP3 was significantly elevated in nasopharyngeal cancer cells, whereas Beclin1 was not significantly changed. Knockdown of BNIP3 expression using small interfering RNA caused LC3-II down-regulation. Conclusion: SYUIQ-5 induces autophagy in cancer cells. This may be related to SYUIQ-5-mediated p-Akt down-regulation and FOXO3a nuclear translocation, which promot LC3 transcription. BNIP3 is involved in SYUIQ-5 induced autophagy.

Key words: autophagy, LC3, BNIP3, Akt, FOXO3a, SYUIQ-5

Telomeres are natural ends of chromosome in eukaryotic cells, and play important roles in protecting the chromosome ends from degradation and recombination, maintaining chromosome stability and withstanding chromosome ends duplication.[1] Human telomeric DNA are composed of 5′-(TTAGGG)-3′ repeats and a 3′ telomeric overhang,[2] and the G-rich DNA strand may form a G-quadruplex. SYUIQ-5 is a cryptolepine derivative with indole and quinoline structures, and our previous studies have shown that it could interact with and stabilize the G-quadruplex,[3] and inhibit the activities of c-myc promoter and telomerase.[4,5]Autophagy is a highly conservative cell behavior in evolution, and is a cell self-degradation process under the pressures of energy deficiency,

metabolism, and so on. Autophagy can not only maintain cell homeostasis, but also promote cell survival. Over upregulation of autophagic activity also leads to cell death, i.e. autophagic cell death (also called as Type II programmed cell death). Many studies suggest that autophagy plays a key role in cell growth, development and diseases occurrence. Our previous studies have demonstrated that SYUIQ-5 -induced autophagy and cell death could be inhibited by the knockdown of autophagy -related gene ATG5, which suggests that SYUIQ -5 could induce autophagy to promote death of tumor cells. In the present study, we investigated the autophagy induced by SYUIQ-5 and its mechanisms.

Materials and Methods

Cell culture. Human nasopharyngeal carcinoma cell line CNE2, CNE1, HONE1 were preserved at by our research center. Cells were cultured in RPMI-1640 medium containing 10% bovine serum, 100 μg/mL ampicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine at 37℃ in an atmosphere of 5% CO_2. Cells in the logarithmic phase of growth were used to perform all experiments in this study.

Materials and reagents. Fetal bovine serum was the product of Gibco Company; Chemiluminescent reagent(ECL), Beclin1 and FOXO3a antibodies were purchased from Cell Signal Company; Akt, p-Akt, BNIP3, GAPDH and HRP -labeled secondary antibody were the products of Santa Cruz Company; LC3 antibody was purchased from Novus Biology Company; cell lysis buffer was from Upstate Biotechnology Company; the test kit for testing the concentration of BCA protein was purchased from Pierce Company; M -MLV was from Promega Company; PCR reaction reagents, Taq DNA polymerase were purchased from Shanghai Shenneng Bocai Company; Trizol reagent was from Invitrogen Company; siRNAs were synthesized by Shanghai GenePharma Company; Primers were synthesized by Invitrogen (Shanghai) Company; Cryptolepine derivative SYUIQ -5 were synthesized by School of Pharmaceutical Sciences at Sun Yat-sen University; SYUIQ -5 was dissolved in 100% DMSO at a final concentration of 50 μg/mL, and kept at -20℃.

Western blot. Cells were collected and washed with PBS twice. After adding100 μL cell lysis buffer, cells were centrifuged at 12,000 rpm/min for 15 min. Then, the concentrations of the proteins were measured using the BCA protein assay kit. Proteins (20 μg) were mixed with sample loading buffer and denatured at 95℃for 10 min. Subsequently, proteins were separated by 10%-15% SDS-PAGE and then transferred onto a nitrocellulose membrane by electrotransfer. The membrane was blocked with TBS-T containing 5% fat-free milk powder, and then was incubated with primary antibodies and secondary antibodies in turn at room temperature for 2 h. The membrane was rinsed in TBST buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% Tween20) for three times (10 min for each time). Finally,the membrane was incubated with ECL reagent and *X* -ray films were developed in dark room.

Reverse transcription-polymerase chain reaction (RT-PCR). Cells with drug treatment were collected and washed with PBS twice. Trizol (1 mL) was added, and then total RNAs were extracted according to protocols in the instruction handbook. In the reverse transcription step, total RNA (2 μg) was mixed with 0.5 μg olig (dT), and then DEPC water was supplemented to the mixture at a final volume of 15 μL. After incubation at 70 ℃ for 5 min, the mixture was placed in the ice bath, and then was successively supplemented with 5 μL 5 ×M -MLV reaction buffer, 1.25 μL 4×dNTP (10 mmol/L), 1 μL M-MLV(200 unit/μL), 0.625 μL RNaseOUTTM (40 u/μL), the suitable volume of DEPC water at a final 25 μL. The reverse transcription reaction was performed by incubation at 42 ℃ for 60 min, and then terminated by incubation at 75 ℃ for 10 min. The PCR reaction was carried out in a total volume of 25 μL comprising 2.5 μL 10 ×PCR buffer containing $MgCl_2$, 0.5 μL 4 ×dNTP (10 mmol/L), 1 μL cDNA, 0.5 μL forward primer, 0.5 μL reverse primer, 0.25 μL *Taq* DNA polymerase and 19.75 μL ddH_2O. PCR was performed as follows: denature at 95°C for 5 min, followed by 30-35 cycles of 95C for 30 s, annealing at 55-68°C for 40 s and extension at 72°C for 60 s, and then a final extension at 72℃ for 10 min. The amplified PCR products were separated by 1.2% agarose gel electrophoresis and examined under UV light. LC3 primer sequences: forward 5′-GTTGCTGACTGACCCTCCA-3′ ;reverse 5′-CGTCTTTCTCCTGCTCGTAG -3′. BNIP3 primer sequences: forward 5′-GAAACAGATACCCATAGCA-3′; reverse 5′- GAACGCAGCATTTACAGA-3′. GAPDH primer sequences: forward 5′-

CCACCCATGGCAAATTCCATGGCA–3′; reverse 5′–TCTAGACGGCAGGTCAGGTCAGGTCCACC–3′.

Immunofluorescence. Cells in the logarithmic phase of growth were collected and diluted at a density of 3×10^5 /mL, and then cells were plated in the 24–well plate containing a glass slide in each well. After 24 h, cells in each well were treated with related drugs. Then the glass slides in each well were taken out and washed with PBS twice. Cells on glass slides were fixed for 20 min using 4% paraformaldehyde, and were incubated with 0.1% TritonX–100 for 10 min. The glass slides were washed with PBS three times. After blocking at room temperature for 1 h using blocking buffer, cells on glass slides were incubated with primary antibody FOXO3a at room temperature for 2 h. After washing with PBS three times again, cells were further incubated with Rhodamine–labeled anti–rabbit secondary antibody in dark at room temperature for 1 h. After washing with PBS three times again, cell nucleus was stained with DAPI, and then the slides were sealed by 90% glycerol. Finally, cells on glass slides were observed by confocal microscope.

RNA interference. CNE2 cells in the logarithmic phase of growth were collected and diluted at a density of 5×10^5 /mL with serum free RPMI–1640 medium, and then cells were plated in six–well plates. When cells grew to 50%–70% confluence, medium in each well were removed, and siRNA transfections were performed using the transfection reagent at a final concentration of 100 nmol/L siRNA in each well. After culture for 6–8 h, transfection complexes were removed, and then RPMI–1640 medium containing the drug was added. After further culture, cells were collected for related assays. siBNIP3 sequences: sense strand 5′–GCUACUCUC AGCAUGAGAAtt–3′; antisense strand 5′–UUCUCA UGCUGAGAGUAGCtg–3′.

Results

SYUIQ–5–induced autophagy and its influence on expression levels of autophagy–related protein Beclin–1 and BNIP3. After CNE2, CNE1 and HONE1 cells were respectively treated with 0.25–2 μg/mL of SYUIQ–5 for 48 h (0.1% DMSO as control group), western blot results showed that the expression levels of LC3 (LC3–I and LC3–II) were all increased. Moreover, the expression level of LC3–I protein was higher than that of LC3–I. In addition, the expression level of BNIP3 was upregulated with the increased concentration of SYUIQ–5, whereas the expression level of Beclin1 did not significantly change in CNE2 cells (Fig. 1).

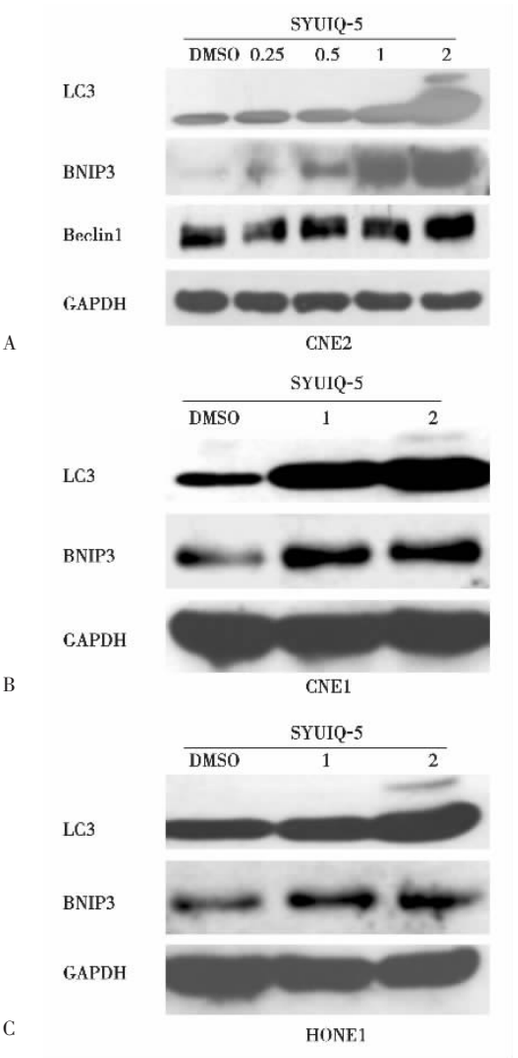

Figure 1 Expressions of LC3, BNIP3 and Beclin1 proteins in CNE2, CNE1 and HONE1 cells treated with SYUIQ-5

Cells were treated with the indicated concentration of SYUIQ-5 (μg/mL) for 48 h.

Influence of SYUIQ–5 on mRNA levels of LC3 and BNIP3. CNE2, CNE1 and HONE1 cells were respectively treated with 0.1% DMSO (as control group) or various concentrations of SYUIQ–5 for 24 h or 48 h (CNE2 cells), or for 48 h (CNE1 and HONE1 cells). The mRNA levels of LC3 and BNIP3 were assayed by RT–PCR. The results demonstrated that the mRNA expression level of LC3 was upregulated but the mRNA level of BNIP3 did not significantly change (Fig. 2).

Influence of SYUIQ–5 on Akt and p–Akt. After

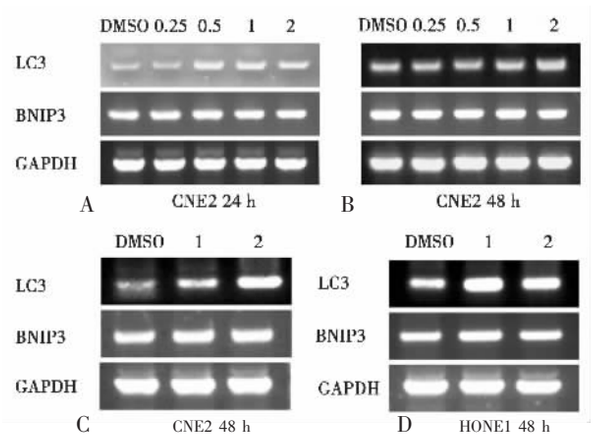

Figure 2 The mRNA expressions of LC3 and BNIP3 in cancer cells treated with SYUIQ-5

CNE2 cells were treated with SYUIQ-5 (μg/mL) for 24 h (A) or 48 h (B); CNE1 (C) and HONE1 (D) cells were treated with SYUIQ-5 (μg/mL) for 48 h, respectively.

CNE2 cells were respectively treated with various concentrations of SYUIQ-5 for 48 h, western blot assay results showed that the expression level of p-AKT was markedly decreased in a dose-dependent manner (Fig.3), but total AKT expression was not influenced.

Immunofluorescence determination of influence of SYUIQ-5 on FOXO3a localization. After CNE2 cells were respectively treated with 0.1% DMSO (control group) and 3.0 μg/mL SYUIQ-5 for 24 h, the changes in localization of FOXO3a were observed by confocal microscopy in cells. The results showed that FOXO3a in control group was mainly distributed in cytoplasm, whereas it was mainly distributed in cell nucleus in the SYUIQ-5 treatment group (Fig. 4).

Influence of inhibiting BNIP3 by RNA interference on autophagy. RNA interference was used to inhibit the expression of BNIP3, and a non-functional RNA fragment was selected as negative control. After siRNA transfection for 24 h, CNE2 cells were respectively treated with 0.1%DMSO (control group) and SYUIQ-5 for 24 h. Western blot assay results showed that the expression of BNIP3 was significantly down-regulated in the siBNIP3 group, which suggested that the expression of BNIP3 was inhibited by siRNA interference. Compared to negative control, the expression of LC3 was also markedly decreased (Fig. 5).

Discussion

Our previous studies have shown that SYUIQ-5,

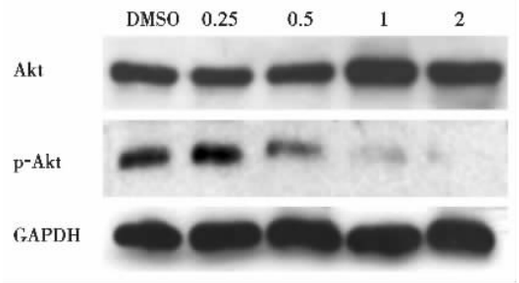

Figure 3 Expressions of Akt and p-Akt proteins in CNE2 cells treated with SYUIQ-5

Cells were treated with the indicated concentration of SYUIQ-5 for 48 h.

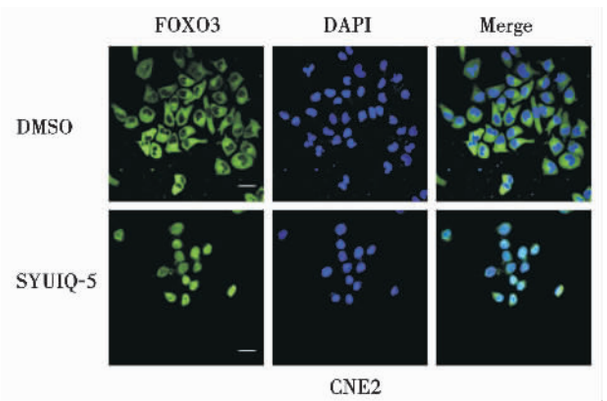

Figure 4 Localization of FOXO3a observed using confocal microscopy in CNE2 cells treated with DMSO or SYUIQ-5 (×40)

CNE2 cells were treated with 0.1% DMSO or 3.0 μg/mL SYUIQ-5 for 24 h. The localization of FOXO3a (green) was observed by confocal microscopy. bars = 40 μm.

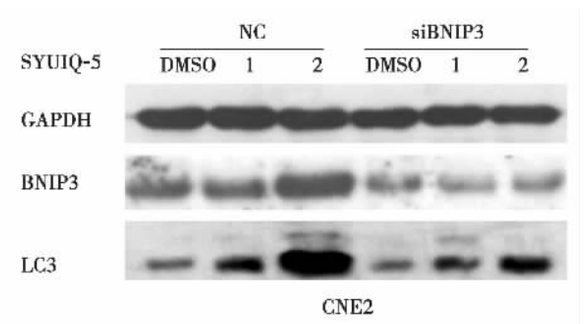

Figure 5 Effect of BNIP3 siRNA on SYUIQ-5-mediated autophagy

a cryptolepine derivative with indole and quinoline structures, could stabilize the G-quadruplex,[3] inhibit telomerase activity in cancer cells,[4] and induce delayed apoptosis.[5] In the present study, it was found that SYUIQ-5 could induce cancer cell autophagy, inhibit the activation of Akt, further induce FOXO3a nuclear translocation, and promote

upregulation of autophagy related BNIP3 gene expression.

Autophagy is a highly conservative cell behavior, and is also an important pathway of substance metabolism in cells. Relative to the ubiqutin–proteasome system which is mainly responsible for degrading short half–life proteins, cell autophagy is considered to be involved in the degradation process of almost all long half–life proteins.[6] In these degradation processes, a great number of vacuoles encapsulating cytoplasm and cell organelle occur in cytoplasm, and the content in vacuoles is also degraded by lysosome. Autophagy is highly associated with cell growth, proliferation and tumorigenesis.

LC3 is the homologue of yeast Atg8 (Aut7/Apg8) gene in mammalian cells. LC3 is located in preautophagosome and the membrane surface of autophagosome, and can serve as a general marker for cell autophagosomal membrane. Newly synthesize LC3 is converted into soluble LC3–I in endochylema. Subsequently, LC3–I is modified by ubiquitination process to bind to phosphatidylethanolamine (PE) on the membrane surface of autophagosome. The binding complex is called LC3–II. The LC3–II content is in proportion to the number of autophagosome, thus, to some extent, the autophagy activity can be inferred according to the change in the content of LC3–II.

After CNE2, CNE1 and HONE1 cells were respectively treated with SYUQ–5, the assay results showed that protein expression levels of LC3– and LC3–I were all increased. Moreover, the expression level of LC3–II protein was higher than that of LC3–. RT–PCR results showed that the mRNA level of LC3 wasalso increased,which suggests that LC3 expression is enhanced at the transcription level after SYUIQ–5 treatment. We propose that SYUIQ–5 could induce the autophagy in cancer cells and upregulate the expression of LC3.

As a member of the Forkhead transcription factor family, FOXO3a can promote the transcription of autophagy–related LC3 gene in skeletal muscle cells. 7 The gene members of Forkhead transcription factor family are important downstream target genes of the PI3K/AKT/PKB signal transduction pathway.[8] As for protein kinase, AKT can regulate cell survival and anti–apoptosis through phosphorylation of a series of substrates. When Akt is not activated, Forkhead proteins generally exist in cell nucleus, and promote the transcription of apoptosis genes. However, after cells are stimulated by various growth factors and further activation of Akt, Akt translocates from the cytomembrane to cell nucleus and phosphorylates FOXO3a. The phosphorylated FOXO3a is transferred outside nucleus and then chelates with cytoplasm protein 14–3–3, which leads to the inactivation of functions regulating the transcription of target genes.[8] Furthermore, the transcription of apoptotic genes can not be promoted, and this leads to cell proliferation. Therefore, FOXO3a mainly exists in endochylema in the situation of cell proliferation. However, FOXO3a is mainly distributed in cell nucleus in the situation of cell apoptosis. To investigate the effect of SYUIQ–5 on the Akt–FOXO3a pathway in CNE2 cells, we treated CNE2 cells with SYUIQ–5. We found that the p–AKT level was downregulated, but the total AKT expression was not influenced. The localization of FOXO3a (green) was observed under confocal microscopy in cells treated with 0.1% DMSO (control group) or SYUIQ–5. In the control group, FOXO3a was mainly distributed in cytoplasm, whereas it was translocated to cell nucleus in the SYUIQ–5 treatment group (Fig. 4). We suggest that SYUIQ–5 could induce FOXO3a nuclear translocation by inhibiting the activation of Akt in CNE2 cells, and further promote LC3 transcription to enhance LC3 expression and finally induce autophagy in cancer cells.

Beclin1 and BNIP3 are important regulators in the process of autophagy. Mammalian Beclin1 is the homologue of yeast Apg6/Vps30 gene. It has been reported that Beclin1 is an important positive regulator in autophagy.[9] As a member of the BH3–only sub–family in the bcl–2 family, BNIP3 belongs to pro–apoptotic protein with the structural domain and transmembrane domain of BH3. BNIP3 can promote the opening of mitochondrial permeability transition pore (MPTP) and mitochondrial damage to induce apoptosis. In addition, the expression of BNIP3 is influenced by some factors, such as hypoxia. Vande Velde et al.[10] firstly reported that BNIP3 induced cell death is associated with the occurrence of autophagic phenomenon. Moreover, the high expression of BNIP3 could lead to autophagy of glioma cells.[11] Although some studies suggest that BNIP3 induced cell death is associated with autophagy, related mechanisms remain unclear. In CNE2, CNE1 and HONE1 cells treated with SYUIQ–5, the expression level of BNIP3 protein was markedly increased, but the mRNA levels of BNIP3

did not significantly change. These suggest that SYUIQ-5 induces high expression of BNIP3 during posttranscriptional regulation. After inhibition of BNIP3 expression by siRNA interference in CNE2 cells, the LC3 level was also decreased, suggesting that BNIP3 plays a key role in SYUIQ-5 induced autophagy.

Currently, whether autophagy is a barrier blocking tumorigenesis or a tumor self-protection response is controversial. In the present study, we found that SYUIQ-5, a G-quadruplex targeting small molecular compound, could inhibit Akt activation to induce nuclear translocation of FOXO3a, and then promote LC3 transcription. It may be one of the important mechanisms that SYUIQ-5 induces autophagy in cancer cells. Also, BNIP3 is involved in SYUIQ-5 induced autophagy. This study provides theory basis for further characterization of the anticancer activity of the G-quadruplex ligand SYUIQ-5 in vitro. However, it needs to be validated that in the autophagy induced by SYUIQ-5 in cancer cells, whether FOXO3a directly participates in the upregulation of LC3, or whether any other transcription factors are involved in autophagy. Moreover, the mechanism that BNIP3 takes participation in autophagy still remains unclear. Further study is required to investigate the anticancer activity of the promising tumor-targeted therapeutic agent SYUIQ-5 *in vivo*.

bReferences

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