Proteasome Inhibitor Drives an Anti-tumor Effects of Human Adenoid Cystic Carcinoma: Correlate with the Emerging Role of Nrf2/Keap1 Signaling Pathway

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Research

Keywords: adenoid cystic carcinoma, proteasome inhibitor, MG132, Nrf2/Keap1, apoptosis

Posted Date: November 18th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-106940/v1>

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Abstract

Background Adenoid cystic carcinoma (ACC) is one of the most common malignant salivary gland tumors. Its unique biological characteristics and complex structures contribute to its poor survival rates. Recently, proteasome inhibitors have shown to elicit satisfactory therapeutic effects in the treatment of certain solid tumors, but few studies have been implemented to investigate the effects of proteasome inhibitor therapy for ACC.

Methods In this present study, cell counting kit-8 assay and flow cytometry assay were performed to determine the effects of proteasome inhibitor (MG132) on cell viability and apoptosis. We applied western blot and immunofluorescence staining to explore the expression of Nrf2/Keap1 pathway and P62, and utilized Nrf2 inhibitor (ML385) to evaluate the role of Nrf2/Keap1 pathway in MG132 induced cell apoptosis.

Results We discovered that MG132 significantly suppressed the growth of ACC-83 cells and induced apoptosis. The application of MG132 induced the up-regulation of Nrf2/Keap1 pathway. Furthermore, inhibition of Nrf2 attenuated the therapeutic effects of MG132 for ACC.

Conclusion Our results revealed that proteasome inhibitors might potentially be useful as a promising agent in cancer chemotherapy to treat ACC through activating Nrf2/Keap1 pathway.

1. Introduction

Salivary gland ACC is known as one of the most unpredictable malignancies of all head and neck malignancies which accounting for 10% of all such tumors.[1, 2] The 5-year survival rate of patients with ACC is lower than 20%. At present, the most routine treatment was surgical excision combined with postoperative radiotherapy. [3]

Protein degradation is critical for protein homeostasis and cell survival because it can replenish the amino acid pool, thereby reconstituting protein synthesis and enabling cells to adapt to changing intracellular and extracellular environments. [4] The proteasome is in connection with the degradation of intracellular proteins specially the protein responsible for the control of cell cycle and the regulation of apoptosis and proliferation.[5]The theory of inducing apoptosis has given rise to the use of proteasome inhibitors in the treatment of tumors.

Proteasome inhibitors can inhibitor cell proliferation and lead cell apoptosis in different tumor cell types. [6] MG132 is a type of proteasome inhibitor which commonly used to investigate the proteasome activity in a range of cell types.[7] Previous trials already proven that MG132 had received the support that the therapeutic potential of the proteasome inhibitors in some types of human carcinomas.[8] However the application of proteasome inhibitors in the treatment of adenoid cystic carcinoma requires further exploration as it is located in the treatment of other solid tumors.

In recent studies, it has been demonstrated that somatic mutations in Nrf2 and Keap1 were identified in some tumors such as lung, head and neck tumors, resulting in sustained activation of Nrf2 and induction of Nrf2 target genes such as cytoprotective enzymes, antioxidant proteins which has the ability to antioxidative stress and anti-cancer agents. [9] Under normal conditions, Nrf2 is invariably ubiquitinated through Keap1 in the cytoplasm and then degraded by the proteasome.[10] Reportedly, MG132 upregulated Nrf2 via both de novo protein synthesis and Keap1 degradation, which may influence the apoptosis of lung cancer cells. [11] To investigate what the role of Nrf2 and Keap1 acted in ACC-83 cells is fatal.

In this study, we sought to explore the biological function of MG132 on anti-tumor in ACC-83 cells. Further we investigated the role of Nrf2/Keap1 pathway in ACC-83 cells proliferation inhibition and apoptosis. This present study may shed new light on anti-tumor effects of proteasome inhibitor therapy for human adenoid cystic carcinoma.

2. Materials And Methods

2.1 Cell lines and cell culture

Human adenoid cystic carcinoma cell(ACC-83) was kindly provided by Peking university. The cells were grown at 37°C atmosphere with 5% CO₂ and 95% air. RPMI 1640 medium supplied with 1% penicillin/streptomycin (HyClone) and 10% fetal bovine serum (FBS) (BI,Biological Industries) were used to culture the ACC-83 cells . the medium was changed every 2 days.

2.2 CCK-8 assay

According to the manufacturer's instructions, the cell counting kit-8 (Dojindo)was used to measure ACC-83 cells proliferation. ACC-83 cells were seeded to 96-well culture plates(Corning) with 1×10^4 cells in every well. After incubation for 24h with 5% CO₂ and 95% air at 37°C,the plates were treated with different concentration proteasome inhibitor MG132 which 2.5,5,10,40,70,100μM for 3,6,9,12,18h.Zero setting was the group that without ACC-83 cells. Group incubated with complete 1640 medium was used as the control group. Every plate was set three times. Premix the CCK-8 and serum-free 1640 medium with the proportion of 1:10.After remove the original complete 1640 medium,100μL premixed solution was added to each well, the cultures were incubated at 37°C for 2 h. Spectrophotometer(Thermo, Finland) was used to measure The optical density (OD) of each well at a wavelength of 450nm.The concentration of 40μM MG132 was considered to be better choice for this experiments.

2.3 Western blot analysis

The western blot analysis will prove the protein expression changes of Nrf2/Keap1 pathway and P62 after ACC-83 cells incubation under different concentrations of MG132. ACC-83 cells were rinsed twice

with cold phosphate-buffered saline (PBS)(Hyclone).And then lysed on ice with RIPA buffer(Solarbio) containing protease inhibitor cocktail with the proportion of 100:1 for 5min.The lysed cells were collected and centrifuged at 12,000rpm for 15 min at 4°C.The protein concentrations in whole cell lysates were determined using BCA protein assay kits(Solarbio).The bovine serum albumin was used as a standard. A total of 30 µg protein was calculated by the volume of lysates for each sample. And equal amount of protein were loaded on a 10-12% SDS-PAGE gel (Beyotime) .Gel electrophoresis was performed at 40 V for 30 min and the following is 100V for 1h.Then transferred the separated polypeptides to a 0.45-µm polyvinylidene difluoride (PVDF) membrane at the condition that 220mA for 30-100min.The PVDF membranes were blocked in a 5% non-fat milk-TBST solution(10 mm Tris-HCl, pH 8.0; 150 mm NaCl; 0.05% Tween-20) for at least 60 min at room temperature while shaking. Then washed the membrane 5 times for 7min each time with TBS-0.05% Tween-20(TBST). Subsequently incubated overnight at 4°C with primary antibodies against Nrf2 (abcam)and Keap1 (proteintech)and P62(cst, [Cell Signaling Technology](#)).After washed 3 times with TBST the membranes were incubated with peroxidase-conjugated secondary antibodies(proteintech)for 60min with shaking. GAPDH(1: 1000 dilution, proteintech)was detected in the same membrane to ensure equal protein loading. Protein bands were visualized by Amersham Imager 600.

2.4 Quantitative real-time polymerase chain reaction (RT-PCR)

ACC-83 cells were seeded in 6-well plates at a density of 1×10^5 cells/well, and incubated with 1640 as the control, MG132($10 \times 40 \times 70 \mu\text{M}$)as trial group for 12h. Total RNA from ACC-83 cells after incubated with MG132 was extracted using RNAiso reagent(takara) according to the manufacturer's instructions. First-strand cDNA was synthesized using PrimeScript™ RT Reagent kit Reverse Transcription System. Real-time PCR was performed with a Roche Light Cycler 480 device in a reacting system that total volume of 20 µl. The cycling parameters used were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s, and a dissociation program of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Every independent experiment was performed in triplicate.

2.5 Flow cytometry

ACC-83 cells were seeded in six-well plates with a density of 6×10^4 cells per well and incubated for 24 h. The cells were treated with MG132 at concentrations of 10, 40, or 70 µM. Conditions respectively for 12 h. The detection of cell apoptosis was through the Annexin V-FITC apoptosis detection kit. The ACC-83 cells were harvested and rinsed twice with cold PBS, trypsinized, and then washed twice by PBS . resuspended in binding buffer and collected into FACS tubes. The standard protocol tubes were successively stained with Annexin V-FITC (5µL) and PI (5µL), and the other tubes stained with both Annexin V-FITC (5µL) and PI (5 µL) as the experimental groups. And then analyzed with flow cytometry.

2.6 Cell immunofluorescence

ACC-83 cells were seeded to a 24-well plate with the coverslip. Treated with proteasome inhibitor MG132 for 24h when the cell reached a confluence of 60~70 % per plate. The cell washed twice by PBS and fixed with 4% paraformaldehyde solution for 1 hour in the indoor temperature, following rinsed thrice by PBS. And then permeabilized and blocked with PBS which containing 0.5% Triton X-100 and blocked with 1 % bovine serum albumin PBS for 20 minutes at RT. Rinsed thrice by PBS the same with the former. Cells were incubated with primary antibody against Nrf2 (cst, [Cell Signaling Technology](#)) and Anti-SQSTM1/p62 both at a dilution of 1:100 at 4 °C overnight. After PBS washed thrice the 488(abcam) and 594(abcam) secondary antibodies were incubated the same conditions with primary antibody for 1 hour in the 37°C. Cell washed thrice with PBS. Following DAPI solution was added. Before imaging cells were rinsed thrice for 5 min per wash. Fluorescence images were captured by a Leica digital microscope. Images were merged using Adobe Photoshop CS6, and no other modifications were made.

2.7 Statistical analysis

All analyses were performed using graphpad prism 8.0 software. Differences between groups were analyzed using two-tailed Student's *t*-test. In all of the analyses, $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1 The effects of MG132 on the viability of ACC-83 cells

As shown in Fig1, to determine whether the MG132 exerted its antiproliferative activity on the ACC-83 cells, we evaluated the effect of MG132 on ACC-83 by CCK8 analysis, as concentration of MG132 increased, the proliferation of ACC-83 cells was significantly inhibited. What's more, compared with the control group, after stimulated for 12h, ACC-83 cells showed a state of inhibition of proliferation at concentrations of 10, 40, and 70 μM MG132. In general, These preliminary results showed that MG132 exerts anti-proliferation activity in ACC-83 cells. However we still do not understand the specific mechanism which will be carried out after MG132 stimulated ACC-83, in the following experiments.

3.2 The effects of MG132 on the cell apoptosis of the ACC-83 cells

Based on the former data, we further more measure the apoptosis of ACC-83 cells. ACC-83 cells were incubated for 12 hours at 10, 40, 70 μM MG132. In order to demonstrate the change of apoptosis, ACC-83 cells, as assessed by flow cytometric analysis. As proved in Fig2, A significant decreasing in the number of both Annexin V-positive and PI-negative (early apoptosis) and Annexin V- and PI-positive (late

apoptosis) ACC-83 cells were detected in the trial group incubated with MG132, compared with the control group. In total, a distinctly increase in apoptotic populations in MG132 group compared to control group in ACC-83 cells.

3.3 Nrf2/Keap1 signaling pathway was activated by MG132

The former studies indicate that the Nrf2/ Keap1 signaling pathway is involved in the oxidative stress of cells and is responsible for the resistance of certain cells and the survival of cancer cells. It has been documented that the Nrf2/ Keap1 signaling pathway may be responsible for the survival of cancer cells and may also induce apoptosis in cancer cells. P62 as an emerging regulator of Nrf2/ Keap1 signal pathway. [12] The DGR and CTR domains of Keap1 (DC domain of Keap1), are responsible for interaction with p62.[13] P62 may act an unexpected role in this study. The existence of such a binary may be due to the intensity and duration of stimulation received by the cells. [14] The total mRNA and protein expression alterations of Nrf2 and Keap1 were detected using western blotting. As shown in Fig3, after stimulated with different concentrations of MG132, the mRNA and protein expression of Nrf2 was significantly increased relative to the control group. On the other hand, the mRNA and protein expression of Keap1 and P62 were significantly reduced relative to the control group. As shown in Fig4, Immunofluorescence analysis of Nrf2 and Keap1 revealed the expression level of Nrf2 was significantly upregulated meanwhile the Keap1 was downregulated. This result indicated that Nrf2/ Keap1 pathway was activating during MG132 stimulated ACC-83 cells.

3.4 Inhibition of MG132 activated Nrf2 could weaken the apoptotic effects of MG132 in ACC-83 cells

Previous studies showed that Nrf2/ Keap1 pathway was activated during MG132 stimulated ACC-83 cells. In order to investigate whether the Nrf2/ Keap1 pathway was related to the apoptosis of ACC-83 cell, ACC-83 cells were incubated with both the Nrf2 inhibitor ML385 and MG132 for 12h to observe its proliferation and apoptosis. As shown in Fig5, ACC-83 cells were treated with the concentration 10, 40, 70 μ m of MG132, and both of the MG132 and Nrf2 inhibitor were added to another group. Incubated for 12 hours the same. The CCK8 assays showed that the survival rate of the cells in the group which added Nrf2 inhibitor was higher than that of the control group. Therefore, we can conclude that the anti-proliferation induced by MG132 after stimulation of ACC-83 cells may be related to the Nrf2/ Keap1 signaling pathway.

Flow cytometry experiments were further used to demonstrate that MG132 induced apoptosis in ACC-83 cells was associated with the Nrf2/Keap1 signaling pathway. Flow cytometry confirmed a decrease in apoptosis rate compared to the control group after co-cultured with the Nrf2 inhibitor.

4. Discussion

Due to the particular biological characteristics and complex structure of ACC, it often recurs after radiotherapy and has a serious radiotherapy side effects. It has series of characters that difficult to cure and local recurrences which always accompany with distant metastases. Although improved CT and MRI make the clinical diagnosis of the ACC to become more easier, survival of ACC patients remains dismal due to the lack of adequate therapies.[15] Therefore, it is very necessary to study new therapeutic strategies to improve the therapeutic efficiency of adenoid cystic carcinoma.

The proteasome is a ubiquitous enzyme complex that plays a key role in protein degradation involved in cell cycle regulation, apoptosis and angiogenesis.[16, 17] Before our study, several researchers have proven that proteasome inhibitors may reduce cell proliferation and induce apoptosis in cancer cells in vitro experiment.[18] And proteasome inhibitors have been demonstrated acts a significant role which decrease proliferation and induce apoptosis in solid and hematological malignancies through a variety of mechanisms, including stabilization of cell cycle regulators and pro-apoptotic factors, and induction of apoptosis.[19] Some have been clinically proven to be able to effect in the treatment of hematological and solid malignancies.[20] In previous studies, proteasome inhibitors have a beneficial effect on human hepatoma cells, follicular lymphoma cells, and solid tumors.[21] There are a substantial number of trials showed that proteasome inhibitor exerts anti-proliferation and induces apoptosis activities. Such as the proteasome inhibitor bortezomib which simultaneously enhances the activity of chemotherapy and radiation in a variety of solid malignancy models both in vitro and in vivo.[22, 23] However, the study of proteasome inhibitors in ACC-83 cells is relatively scarce, and its mechanism is not completely understood. Therefore, in this study, different concentrations of the proteasome inhibitor MG132 was utilized to stimulate ACC-83 cells, and the proliferation and apoptosis of ACC-83 cells were observed. The data showed that ACC-83 cells showed the condition that proliferation inhibition and induced apoptosis after incubated with MG132.

The activation of multiple anti-apoptotic and pro-proliferation signaling pathways in cells requires the involvement of proteasome such as the Nrf2/ Keap1 signaling pathway[24], which interferes with the original cell proliferation, differentiation and apoptosis process by inhibiting the activity of proteasome, thus the theory of inducing apoptosis promotes the strategy of using proteasome inhibitors to treat tumors. To explore the mechanism of AMC cell proliferation inhibition and apoptosis induced by MG132, Western blotting analysis and RT-PCR were used to investigate the expression of Nrf2/ Keap1 in the ACC-83 cell which was incubated with MG132. As some studies show that p62 played an important role in regulating Nrf2/ Keap1 signal pathway.[25] P62 interacts with the Nrf2-binding site on Keap1, so that Nrf2 release from Keap1. [26] P62 and Nrf2 regulate each other's expression. Our data showed that the mRNA and protein expression of p62 and Nrf2 increased significantly while the Keap1 decreased in the ACC-83. The result of transmission electron microscope also proved that. All of these identified MG132 as being able to inhibit ACC-83 cell proliferation and induce apoptosis, and may be through the Nrf2/Keap1 pathway. Further studies are needed to use different cell lines in genetic and animal in vivo tests, and the detailed mechanism of MG132 in ACC-83 cells needs to be more fully understood. Taken together, the present study may provide a reference for clinical applications.

Conclusion

The present data indicate that the proteasome inhibitor MG132 inhibits the proliferation of adenoid cystic carcinoma cells and induces apoptosis in vitro. Our study found that the mechanism by which MG132 inhibits proliferation and induces apoptosis in ACC-83 cells may be correlated with Nrf2/ Keap1 signaling pathway. Consequently, Proteasome inhibitor treatments provided a new perspective on ACC.

Declarations

Acknowledgments

N/A

Authors' contributions

Dongsheng Zhang and Shengyun Huang conceived and designed the survey. Haiwei Wu and Zhanwei Chen supervised and administrated the survey. Jiatong Sun and Zhiyuan Gong response to the methodology. Enli Yang and Xiaoya Lu were involved in the data collection and analysis. Jiazhi Xu wrote the manuscript. The authors have read and approved the final manuscript.

Funding

The present study was supported by grants from the Shandong Provincial Natural Science Foundation (grant no. 2016ZRB1462), National Natural Science Foundation of China (grant number 81901022), China Postdoctoral Science Foundation (grant no. 2019M652380), Shandong province medical and health science and technology development plan project (grant no. 2017WSB01006), Shandong Provincial Natural Science Foundation (grant no ZR2017BH069).

Availability of data and materials

The datasets are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests The authors declare that

The authors declare no conflicts of interest.

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Figures

Figure 1

MG132 inhibited the proliferation of ACC-83 cells. A. ACC-83 cells were treated with MG132 (0, 2.5, 5, 10, 40, 70, 100 μM) for 12h. B. Cells were treated with 40 μM MG132 for 3, 6, 9, 12, 24, 36h. Final concentrations of the compounds were as shown. Cell viability was determined by CCK8 assay (mean $\pm$ SD). All data was obtained from ten independent experiments performed in triplicate. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus control.

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* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control.

Figure 2

Assessment of apoptosis by annexin V/PI and flow cytometry in ACC-83 cells treated with MG132 (10, 40, 70, μ M) for 12h. ACC-83 cells untreated were used as control. As the concentration of the drug increased, the apoptosis rate of ACC-83 cells increased. * $P < 0.05$, ** $P < 0.01$ versus control.

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Figure 3

MG132 induced inhibition of ACC-83 cells proliferation through activating Nrf2/Keap1 signaling pathway. A. Cells were treated with 0 μ M, 10 μ M, 40 μ M, 70 μ M MG132 12h. Western blot analysis of the protein expression of Nrf2, Keap1 and P62 between the control. All data were obtained from independent experiments. B. The protein and mRNA expression of Nrf2 was significant increased while the protein and

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Figure 4

Immunofluorescence analysis of Nrf2 and Keap1 in ACC-83 treated with MG132(40μM) for 12h. A.B. Nrf2 and Keap1 punctate dots were visualized by fluorescence microscopy.

Figure 4

Immunofluorescence analysis of Nrf2 and Keap1 in ACC-83 treated with MG132(40 μ M) for 12h. A.B. Nrf2 and Keap1 punctate dots were visualized by fluorescence microscopy.

Figure 5

ACC-83 cells were incubated with both the Nrf2 inhibitor ML385 and MG132 for 12h to observe its proliferation and apoptosis. A. cell viability was determined by CCK8 assay (mean±SD). All data were obtained from three independent experiments performed in triplicate. B.C. Cells were treated with 0μM, 10μM, 40μM, 70μM MG132 12h. C. Cells were treated with both ML385 and MG132, the data was measured by flow cytometry. The apoptosis of group incubated with MG132 was higher than the group

incubated both ML385 and MG132. D.showned cell death% of group cell treated with MG132 and the group that treated with both MG132 and ML385. * $P < 0.05$,** $P < 0.01$ versus group treated with MG132.

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