Ketogenic diet promotes apoptosis of tumor cells and inhibits tumor growth through inhibiting Wnt1/β-catenin signaling pathway in mouse xenograft models of human colon cancer

Kai Wang
Shenzhen Baoan District Central Hospital
Zhen-Xiong Qiu
Shenzhen Baoan District Central Hospital
Teng-Fei Wu
Shenzhen Baoan District Central Hospital
Si-Zhong Chen
Shenzhen Baoan District Central Hospital
Min Xiao
Shenzhen Baoan District Central Hospital
Si-Hui Huang (huangsihui12@163.com)
Shenzhen Baoan District Central Hospital
Rong-Jiang Li (lirongjiang12@163.com)
Shenzhen Baoan District Central Hospital

Research

Keywords: Ketogenic diets, colon cancer, subcutaneous tumor, apoptosis, Wnt1/β-catenin signaling pathway

Posted Date: August 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-58634/v1

Abstract

Background: High-fat, low-carbohydrate and adequate-protein ketogenic diets (KD) are expected to become an effective adjunct therapy for cancer patients. However, the direct effects of KD on tumor cells and the underlying mechanisms are elusive. In this study, the nude mouse models of subcutaneous transplanted human colon cancer cells were established and applied to study the effects and mechanisms of KD on the growth of subcutaneous tumors in nude mice.

Methods: Male nude mice were injected subcutaneously with human colon cancer HCT-116 cell line to construct a subcutaneous tumor model of human colon cancer. The successfully constructed subcutaneous tumor mice were divided into normal diet group and KD group. The mice were anesthetized and euthanized after 30 days of feeding, the subcutaneous tumor tissues were collected, and the size of tumors was measured and weighed. HE staining was used to observe the pathological changes of subcutaneous tumor tissues in normal feeding group and KD group. TUNEL staining was used to detect the level of apoptosis in tumor tissue. Immunohistochemistry of subcutaneous tumor tissues was used to detect the expression levels of Wnt-1 and β-catenin. In addition, RT-qPCR and western blotting were applied to detect the expression levels of Wnt1/β-catenin signaling pathway-related proteins.

Results: After 30 days of normal diet and KD feeding, the subcutaneous tumor tissues of human colon cancer mice were taken out for various assays. The results of tumor size measurement showed that the tumor size and weight of KD group were significantly smaller than that of the normal diet group. HE staining showed that the pathological characteristics of colon tumor tissue in the KD group were significantly improved, and the infiltration of inflammatory cells was reduced. TUNEL staining showed that the apoptosis level of tumor cells in the KD group was significantly increased compared to the normal diet group. RT-qPCR and western blotting revealed that the expression of pro-apoptotic proteins such as caspase 3、caspase 9 and Bax were increased（$P < 0.01$）, while the expression of anti-apoptotic protein such as Bcl-2 or survivin was decreased （$P < 0.01$）. Furthermore, the expression of Wnt1/β-catenin signaling pathway-related proteins including Wnt1 and β-catenin were largely reduced after 30 days of KD feeding compared to normal feeding group （$P < 0.01$）.

Conclusions: Ketogenic diets (KD) promotes apoptosis of human colon cancer subcutaneous tumor cells and inhibits the growth of tumor by inhibiting Wnt1/β-catenin signaling pathway in mouse subcutaneous tumor models of human colon cancer.

Background

Colon cancer is one of the most common gastrointestinal malignant tumors in the world and it is also one of the main causes of cancer death in western developed countries [1]. The 2018 Global Cancer Report released by the International Cancer Research Center shows that colon cancer ranks fourth in morbidity and second in mortality worldwide [2]. In the past few decades, the incidence of colon cancer in China has increased rapidly. According to 2015 China Cancer Statistics, the incidence and mortality of

colorectal cancer in China ranks fifth among all malignant tumors [3, 4]. The present study suggests that low fiber diet, family history, drinking, mental problems and other factors are the main risk factors for colon cancer [5, 6]. Radical surgery is the predominant treatment for stage I to III colon cancer, but most patients still need postoperative chemotherapy. Although chemotherapy has a good inhibitory effect on the growth of tumor, its severe toxic and side effect are widely recognized [7, 8]. Because of this, more and more adjuvant support therapies that can inhibit tumor growth and reduce the side effects of chemotherapy are being developed and applied, such as ketogenic diets.

High-fat, low-carbohydrate and adequate-protein ketogenic diets (KD) was originally used for the treatment of epilepsy [9, 10]. Recent studies revealed that KD can also be applied to the treatment of some metabolic or endocrine diseases such as obesity, diabetes and hypertension [11, 12]. As we know, malignant tumors were also regarded as a metabolic disease due to their altered glucose metabolism, which was tumor cells preferentially utilize glycolysis instead of oxidative phosphorylation to generate energy and other metabolites [13]. Therefore, KD is becoming a promising adjuvant support therapy for cancer treatment in recent years [14]. Indeed, it has reported that KD has been used in the adjuvant treatment of many different tumors, such as gastric cancer, prostate cancer or glioma, and had a positive therapeutic effect while no obvious adverse effects were observed [15–18]. Further studies showed that KD can limit the growth of tumor, enhance the effects of chemotherapy to tumor cells while reducing the toxicity of chemotherapy to normal cells [19], and attenuate the inflammatory level of tumor [20]. Although many preclinical studies showed that KD has an acceptable therapeutic effect in cancer treatment, it was a recognized fact that not all types of tumors are sensitive for KD therapy [21]. Therefore, the therapeutic effect of KD on cancer is controversial and detailed molecular mechanisms of KD therapy were elusive up to now [22]. It has reported that KD can significantly limit the growth of human colon cancer subcutaneous transplanted tumors in nude mice [23]. However, the specific molecular mechanism was not clear, which was an important scientific problem to be solved in this study.

In this study, the nude mouse model of subcutaneous transplanted human colon cancer cells was built and applied to study the therapeutic effects and mechanisms of KD on the growth of subcutaneous colon tumors in nude mice. Histopathological observation of subcutaneous colon tumors showed that the size of the tumors was significantly reduced and the apoptosis level of the tumor cells increased after KD feeding. Subsequent study revealed that KD induces apoptosis of tumor cells and inhibits tumor growth through inhibiting Wnt1/β-catenin signaling pathway. These findings revealed that the positive therapeutic effects and possible mechanisms of KD on colon tumors and provided scientific evidence for better understanding the selectivity of KD in tumor treatment.

Methods

Animals

The healthy adult male nude mice (6 week-old weighting 20 to 25 g) were provided by Hunan SJA Laboratory Animal Co., Ltd (License number: SCXK (Xiang 2016-0002). The animals were fed in SPF

animal house of Shenzhen University Health Science Center. Each mouse was housed in a single cage to prevent fighting and had access to food and water ad libitum. The mice were maintained at a constant temperature (23 ± 2°C) and humidity with a 12-h light–dark cycle and were acclimatized to the environment for at least a week before the experiments. All procedures, care, and handling of the rats were approved by the Ethics Committee of Shenzhen University Health Science Center.

Cell lines, Antibodies and Reagents

Human colon cancer cell line HCT-116 (BNCC339915) was purchased from BeNa Bio Technologies Inc (Beijing, China). Complete DMEM medium (KGM12800S-500) and phosphate buffer saline (PBS) (KGB5001) were purchased from KeyGEN BioTECH (Nanjing, China). Ketogenic feed was purchased from Medicience Biomedicine Co., Ltd. (Yangzhou, China). Trypsin-EDTA solution (T1300), eosin staining solution (G1100) and Scott blue/blue liquid (G1865) were purchased from Solarbio Life Sciences (Beijing, China). Hematoxylin Staining Solution (ZLI-9610), mouse anti-GAPDH Monoclonal antibody (TA-08), HRP-conjugated goat anti-rabbit IgG (H + L) secondary antibody (ZB-2301) and HRP-conjugated goat anti-mouse IgG (H + L) secondary antibody (ZB-2305) were purchased from ZSGB-Bio Technologies Inc (Beijing, China). One-step TUNEL apoptosis detection kit (C1088) was purchased from Beyotime Bio Technologies Inc (Shanghai, China). Rabbit anti-beta catenin polyclonal antibody (51067-2-AP) and rabbit anti-Wnt1 polyclonal antibody (27935-1-AP) were purchased from Proteintech (Wuhan, China). Rabbit anti-Bcl-2 polyclonal antibody (A11025) and rabbit anti-survivin monoclonal antibody (A19663) were purchased from ABclonal (Wuhan, China). Rabbit anti-Bax monoclonal antibody (ab32503), rabbit anti-caspase 3 monoclonal antibody (ab197202) and rabbit anti-caspase 9 monoclonal antibody (ab32539) were purchased from Abcam (Cambridge, UK). DAB color development kit (CW0125), neutral resins (CW0136), Ultrapure RNA extraction kit (CW0581M) and TRIzon reagent (CW0580S) were purchased from Cwbio Inc (Beijing, China). HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (R223-01) was purchased from Vazyme Biotech Co.Ltd (Nanjing, China). The 2×SYBR Green PCR Master Mix (A4004M) was purchased from Lifeint Technologies Inc (Xiamen, China). Matrigel (354248) was purchased from Corning BD Biosciences (Mississauga, ON, Canada). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Colon cancer xenograft models

Subcutaneous tumor model of human colon cancer in nude mice was established according to the method described previously [24]. In brief, human colon cancer HCT-116 cells were cultured in DMEM medium containing 10% fetal bovine serum and growth to 90% confluence, then the cells collected by digesting with trypsin and suspended to a concentration of 5×10^6 cells/ml with a mixture of Matrigel and PBS at a ratio of 1:1. Subsequently, the above HCT-116 cell suspension was subcutaneous injected under the skin in the right flank of nude mice (6 week-old male nude mice weighing 20–25 g) with an amount of 0.1 ml/mouse. The growth of tumors was monitored weekly with rulers by measuring the size. The mice were randomly divided into two groups (Normal diet group and ketogenic diets group) once the tumor size reached 0.5cm^3. The mice in normal diet group were fed with standard mouse feed daily, and

the mice in KD group were given ketogenic diets every day. The body weights of nude mice were recorded every two days. After 30 days of ketogenic diet or normal diet, the mice of each group were euthanized by CO_2 inhalation and the subcutaneous tumors were obtained and applied to the following experiments, such as the measurement of tumor size or histopathological examination.

Histopathological examination of tumor

The histological method was used to detect the pathologic changes of subcutaneous tumors and performed as described previously [25]. Briefly, mice from normal diet group and ketogenic diets group were euthanized by CO_2 inhalation on day 30 after ketogenic diets or normal diets. The subcutaneous tumors were harvested and immediately fixed in 4% paraformaldehyde, then embedded with paraffin, and cutted into 3 μm thick slices. The sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) successively. Subsequently, the sections were washed with flowing water, then dehydrated with high concentrations of ethanol and transparentized with xylene, sealed with neutral resins and the histopathological alterations observed under light microscopy.

TUNEL Assay

The apoptosis of tumor cells was determined by TUNEL assay via using a commercial One-step TUNEL apoptosis detection kit (Beyotime) according to the manufacturer's instructions. Briefly, the paraffin embedded tumor sections were deparaffinized with xylene, then rehydrated with a series of concentrations of ethanol. The sections were removed to a wet box and the antigen retrieval was performed by dropping 50 μg/ml of Proteinase K working buffer on the sections and incubated at 37 °C for 30 min. Then the sections were washed three times with PBS and the residual PBS around the tissue sections was removed with absorbent paper. 50 μl of freshly prepared TUNEL test solution (5 μl of TdT enzyme with 45 μl of fluorescence-labeled solution) was added to each tissue section and incubated at 37°C in the dark for 1 h. After incubation, the sections were washed three times with PBS and the residual liquid around the tissue sections was removed with absorbent paper. Then the tissue sections were mounted with anti-fluorescence quenching mounting solution and observed under a fluorescence microscope. The excitation wavelength is 450-500nm and the emission wavelength is 515-565 nm, the green fluorescence can be observed.

Immunohistochemistry

Immunohistochemistry was applied to detect the expression of Wnt1/β-catenin signaling proteins of subcutaneous tumors and performed according to a method described previously [26]. Briefly, the above prepared tumor sections were deparaffinized with xylene and rehydrated with a series of concentrations of ethanol, then antigen repair was performed with 10 mM citrate buffer at pH 6.0. Subsequently, the endogenous peroxidase blocking solution was eliminated by incubating the sections with fresh prepared 3% hydrogen peroxide solution at room temperature for 10 min. Then the sections were washed three times with PBS and blocked in 5% BSA at 37 °C for 30 min to prevent nonspecific antigen-antibody binding. Subsequently, the diluted anti-Wnt1 antibody (1:300) and anti-β-catenin antibody (1:200) were

added to the sections and incubated at 4 °C overnight. After washed three times with PBS, the sections were incubated with diluted peroxidase-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:100) at 37 °C for 30 min. The sections were wash three times with PBS and color development with 3,3 N-Diaminobenzidine Tertrahydrochloride (DAB) for 5-10 min. After washed with PBS, the sections were redyeing with hematoxylin for 3 min, differentiated with hydrochloric acid ethanol differentiation solution and back to blue. At last, the sections were washed, dehydrated, transparentized, mounted and observed under light microscopy.

Real-time quantitative PCR (RT-qPCR)

Real-time quantitative PCR (RT-qPCR) was applied to detect the mRNA expression of caspase 3 and caspase 9 of subcutaneous tumors, which performed as described previously [27]. In brief, the total RNA extraction and cDNA synthesis of subcutaneous tumors were performed by using the commercial kits according to the manufacturer's instructions. The specific primers of caspase 3 and caspase 9 were designed by Oligo 7 software and listed in Table 1. The reaction volume of RT-qPCR was 25μl including 1 μl of cDNA template, 1 μl of forward primer, 1 μl of reverse primer, 12.5 μl of 2×SYBR Green PCR Master Mix and 9.5 μl of RNase Free dH2O. The RT-qPCR reaction condition as follows: predenaturation at 95°C for 10 min，denaturation at 95 °C for 10 sec, annealing at 58 °C for 30 sec and elongated at 72 °C for 30 sec, 40 cycles. GAPDH was as the control. The relative expression of caspase 3 and caspase 9 in the transcript levels were analyzed by $2^{-\triangle\triangle Ct}$ method.

Western Blot

The total proteins of tumors in nude mice were shredded, ground to powder in the presence of liquid nitrogen, and lysed by protein lysate containing protease inhibitor cocktail on ice bath for 30 min. Then the lysate was centrifuged at 12000 rpm/min for 15 min, the collected supernatant was total proteins. The protein concentrations were determined by a commercial BCA quantitative kit according to the manufacturer's instructions. The protein electrophoresis was performed by 12% SDS–PAGE and electrotransferred onto polyvinylidene difluoride membranes. After electrotransferred, the PVDF membrane was blocked in 3% BSA at room temperature for 1 h and incubated at 4°C overnight with diluted anti-caspase 3 antibody (1:1000), anti-caspase 9 antibody (1:1000), anti-Bax antibody (1:1000), anti-Bcl-2 antibody (1:1000), anti-survivin antibody (1:1000), anti-Wnt1 antibody (1:1000), anti-β-catenin antibody (1:5000) and anti-GAPDH antibody (1:2000), respectively. After washed three times with TBST, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibodies (1:5000) at room temperature for 2 h. The protein bands were visualized with the SuperSignal WestPico chemiluminescence substrate.

Statistical analysis

All experimental values were presented as the Mean ± SD. Each experiment was repeated at least 3 times independently. Statistical analysis of data was performed using Graphpad Prism 7.0 software. One-way ANOVA was used to analyse the differences between more than two groups, and non-paired student's *t*

test was used to analyse the difference between two groups. P <0.05 was considered statistically significant.

Results

Ketogenic diets (KD) had no significant effect on tumor size in nude mouse xenograft models of human colon cancer

To explore the effect of ketogenic diets (KD) on subcutaneous tumor in nude mouse xenograft models of human colon cancer, the nude mouse xenograft models were established and fed with normal or ketogenic diets for 30 day. Our findings showed that there was no significant difference in tumor size between normal feeding group and KD feeding group (**Figure 1A**). In addition, tumor weighing revealed that the tumors of nude mice in normal feeding group have a similar weight with KD feeding group (**Figure 1B**). These findings suggested that KD had no significant effect on tumor size and weight in nude mouse xenograft models of human colon cancer.

KD decreased the inflammatory level of subcutaneous tumor in nude mouse xenograft models

Inflammatory infiltration is a typical characteristic of malignant tumor. Here, the inflammatory level of subcutaneous tumor in nude mouse xenograft models was determined by H&E staining. Our findings showed that the tumor cells of subcutaneous tumor in normal feeding group were arranged in a solid shape and some tumor cells had a tubular distribution. Moreover, the tumor tissue had a large number of inflammatory cells invasion and infiltration. Nevertheless, the histopathological characteristics of subcutaneous tumor in KD feeding group were improved significantly compared to normal diets group. Furthermore, the inflammatory cells invasion and infiltration of tumor in KD feeding group were decreased significantly compared to normal diets group **(Figure 2)**. The above findings indicated that KD attenuated inflammatory cells invasion and infiltration and improved histopathological characteristics of subcutaneous tumor in nude mouse colon cancer xenograft models.

KD triggered the apoptosis of tumor cells of subcutaneous tumor in nude mouse xenograft models

The above findings suggested that KD feeding decreased inflammatory cells invasion and infiltration and improved histopathological characteristics of subcutaneous tumor, and ultimately limited the growth of the tumor. So does KD have any effect on tumor cells? Here, TUNEL staining was applied to study the apoptosis of tumor cells of subcutaneous tumor in nude mouse xenograft models. The findings showed that the apoptotic cells are stained green and the nucleus are stained blue, and there were more apoptotic cells appeared in KD feeding group compared to normal feeding group **(Figure 3A).** In addition, the ratio of apoptotic cells was quantified by analyzing the fluorescence intensity of apoptotic and total cells and revealed that KD feeding group had a higher apoptotic ratio of tumor cells than normal feeding group **(Figure 3B)**. To further study the expression of apoptosis-related proteins such as caspase 3 or caspase 9, qRT-PCR was employed and revealed that the expression of caspase 3 or caspase 9 mRNA was increased significantly after the nude mice with KD feeding 30 days compared to normal feeding **(Figure 3C)**. In

addition to caspase 3 and caspase 9, there are other apoptosis-related proteins involved in apoptosis, including pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 or survivin. Therefore, western blot was adopted to detect the expressions of caspase 3, caspase 9, Bax, Bcl-2 and survivin. Our findings showed that the expressions of pro-apoptotic proteins including caspase 3, caspase 9 and Bax were increased significantly after the nude mice with KD feeding 30 days compared to normal feeding, while the expressions of anti-apoptotic proteins including Bcl-2 and survivin were decreased compared to normal feeding group **(Figure 3D)**. To better display the expression differences of caspase 3, caspase 9, Bax, Bcl-2 and survivin in normal feeding and KD feeding group, the gray scale analysis of western blot bands was performed and the similar results were obtained **(Figure 3E)**. Our findings suggested that KD increased the expression of pro-apoptotic proteins and decreased the expression of anti-apoptotic proteins, and then promoted the occurrence of apoptosis of tumor cells. Taken together, these findings indicated that the KD could promote the apoptosis of subcutaneous tumor in nude mouse xenograft models by increasing the expression of pro-apoptotic proteins and decreasing the expression of anti-apoptotic proteins.

KD attenuated the expression of Wnt1/β-catenin signaling proteins and further triggered apoptosis in subcutaneous tumors

The above findings showed that KD inhibited the subcutaneous colon tumor growth in nude mouse by promoting the apoptosis of tumor cells. Apoptosis is a type of programmed cell death and regulated by various signaling pathways, for instance the Wnt1/β-catenin signaling pathway. Here, the expression of Wnt1 and β-catenin were determined by western blot and immunohistochemistry. First, western blot revealed that the expression of Wnt1 and β-catenin were attenuated significantly after the nude mice with KD feeding 30 days compared to normal feeding **(Figure 4A)**. To quantify the difference of protein bands, the gray scale analysis of western blot bands was performed and the similar results were obtained **(Figure 4B)**. In addition, immunohistochemistry also revealed that the expression of Wnt1 and β-catenin were attenuated significantly after the nude mice with KD feeding 30 days compared to normal feeding **(Figure 4C)**. The quantitative analysis to the findings of immunohistochemistry also showed that there was a lower expression of Wnt1 and β-catenin after the nude mice with KD feeding 30 days compared to normal feeding **(Figure 4D)**. These findings indicated that KD inhibited the Wnt1/β-catenin signaling pathway by decreasing the expression of Wnt1 and β-catenin, eventually promoted the apoptosis of tumor cells and limited the growth of subcutaneous tumor in nude mouse xenograft models.

Discussion

As we know, tumor cells are more preferable to utilize glycolysis instead of oxidative phosphorylation to produce energy than normal cells. Therefore, aerobic glycolysis is considered as an important feature of cancer metabolism,which is termed as Warburg effect [28, 29]. Previous studies have shown that the growth rate of tumors is positively correlated with the body's blood glucose concentration [30], namely the higher blood glucose concentration of tumor patients accelerate the growth of tumors. While the size and growth rate were reduced once the blood glucose concentration was decreased. Therefore, the reduced

intake of carbohydrates could effectively cut off the energy supply of the tumor, which limited the growth of tumor. For this reason, low carbohydrate-based diet therapy is becoming a promising supportive therapy in cancer treatment.

Ketogenic diets (KD) are a formula diet composed of high fat, low carbohydrate, moderate protein and other nutrients [31]. In the human body, the fatty acids of KD were oxidized and decomposed to intermediate metabolites including acetoacetic acids, β-hydroxybutyric acids and pyruvic acids in liver, which simulated the physiological conditions induced by exercise or fasting. For this reason, KD has been suggested to reduce seizure frequency of epilepsy patients and was widely used in the treatment of various types of epilepsy [32]. In fact, KD has been developed as a treatment for epilepsy almost for a century for its high safety and small side effects. The following research revealed that KD therapy has gradually been used in the treatment of chronic diseases, such as obesity, various acute and chronic inflammations, autism and tumors [16, 33]. There were four types of KD have been developed up to now, which was long-chain triacylglycerol diet (LCTD), medium-chain triacylglycerol diet (MCTD), modified Atkins diet (MAD) and low glycemic index diet (LGID), respectively. Recent studies showed that KD can significantly inhibit tumor growth, prolong the survival of patients, improve the quality of life of patients, and enhance the sensitivity of patients to chemotherapy [34]. Therefore, KD has been developed a type of adjuvant support therapy and widely used in the treatment of tumor animal models and clinical tumor cases. The mechanisms of KD in cancer treatment were elusive. One possible mechanism was that tumor cells lack specific enzymes that metabolize ketone bodies, which led to a failure in the energy supply of tumor cells and further caused the death of tumor cells. However, the detailed molecular mechanism of KD in cancer treatment was an interesting question worth exploring in future studies.

In this study, the nude mouse xenograft model of subcutaneous transplanted human colon cancer cells was built and applied to study the therapeutic effects and mechanisms of KD on the growth of subcutaneous colon tumors in nude mice. Our findings found that the size of tumor in KD feeding group slightly smaller than the tumor in normal feeding group (Fig. 1), suggested that KD can delay the growth of tumor. This was consistent with the findings of Hao. et al [23]. Subsequent H&E staining showed that KD attenuated inflammatory cells invasion and infiltration and improved histopathological characteristics of subcutaneous tumor in nude mouse colon cancer xenograft models (Fig. 2). The subsequent TUNEL assay showed that KD enhanced the apoptosis of subcutaneous tumor cells, further qRT-PCR and western blot assay revealed that KD significantly increased the expression of pro-apoptotic proteins and decreased the expression of anti-apoptotic proteins, and then caused the apoptosis of tumor cells (Fig. 3). It was worth mentioning that β-hydroxybutyric acid, one of KD intermediate metabolites, which could significantly inhibit the mTOR signaling pathway in HT29, Caco-2 colon cancer cells or C57BL-6 mice, and also upregulate the expression of midgut-specific transcription factors of intestinal cells, thereby maintaining intestinal homeostasis of mice [35, 36]. Therefore, these findings indicated that KD promoted the apoptosis of tumor cells, improved the histopathological characteristics and eventually limited the growth of subcutaneous tumor in nude mouse xenograft models.

Wnt signaling pathway is an important pathway that plays a vital regulatory role in the early embryonic development, the formation of nerve cells and the maintenance of hematopoietic stem cells' self-renewal [37]. There are three types of Wnt signaling pathway including classical signaling pathways, non-classical signaling pathways and planar polar signaling pathways identified up to now. Wnt/β-catenin signaling pathway was the most widely studied pathway and played an important role in maintaining small intestine tissue stability, regulating bone density and other functions [38]. Wnt is a proto-oncogene discovered in 1982 in the study of mouse papillomavirus-induced mouse breast cancer, and is homologous to the Drosophila embryonic developmental gene Wingless, so it is named Wnt. Present studies has identified more than 20 Wnt genes in vertebrates and 16 Wnt genes in humans. Wnt ligands can be divided into two categories based on their activity differences: Wnt1/wg and Wnt5a. The Wnt1 signal can be transmitted to β-catenin after it bound to its receptor frizzled protein (Fz) and LRP5/6, which promoted the dissociation of β-catenin from the complexes of Axin, APC and GSK-3β. The dissociated β-catenin accumulated in the cytoplasm and was transferred to the nucleus to regulate the transcription of genes such as CyclinD1, C-myc and Survivin, et al [39, 40]. It has reported that the occurrence of colon cancer is related to the uncontrolled accumulation of β-catenin in the cells [41]. Indeed, the excessive activation of Wnt pathway will lead to intestinal lesions, in fact, about 90% of colon cancer patients have abnormal activation of the Wnt signaling pathway [42]. It has been confirmed that the abnormal activation of Wnt signal is the predominant reason for the occurrence and development of colon cancer [43, 44]. Therefore, development of Wnt pathway-specific targeted inhibitors will be a promising therapy for colon cancer treatment.

In consideration of the importance of the Wnt/β-catenin signaling pathway in colon cancer, the expression of Wnt1/β-catenin signaling pathway was studied. Our findings showed that KD inhibited the Wnt1/β-catenin signaling pathway by decreasing the expression of Wnt1 and β-catenin. In addition, KD also decreased the expression of anti-apoptotic proteins such as Bcl-2 and survivin and increased the expression of pro-apoptotic proteins such as caspase 3, 9 and Bax. Survivin was the target gene of Wnt1/β-catenin signaling pathway and was also the strongest known anti-apoptotic factor. Therefore, KD inhibited the expression of anti-apoptotic proteins and enhanced the expression of pro-apoptotic proteins by inhibiting Wnt1/β-catenin pathway, which further promoted the occurrence of apoptosis of tumor cells and improved the histopathological features of subcutaneous tumor, ultimately limited the growth of tumor. In conclusion, these findings revealed that KD inhibited the Wnt1/β-catenin signaling pathway by decreasing the expression of Wnt1 and β-catenin, eventually promoted the apoptosis of tumor cells and limited the growth of subcutaneous tumor in nude mouse xenograft models.

Conclusions

Ketogenic diets (KD) promotes apoptosis of human colon cancer subcutaneous tumor cells and inhibits the growth of tumor by inhibiting Wnt1/β-catenin signaling pathway in mouse subcutaneous tumor models of human colon cancer.

Declarations

Ethics approval and consent to participate

All procedures, care, and handling of the rats were approved by the Ethics Committee of Shenzhen University Health Science Center.

Consent for publication

Not applicable.

Availability of data and materials

Data is available from the authors by request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work were supported by grant from Science and Technology Bureau of Baoan District of Shenzhen city.

Authors' contributions

K.W. and R.L. conceived and designed the experiments and analyzed results. Z.Q., T.W., S.C., and M.X. carried out most of the experiments, including western blotting detection, immunohistochemistry and qRT-PCR experiments. K.W., Z.Q. and S.H. participated in data analysis. K.W. and R.L. wrote the paper. Z.Q. and S.H. edited the manuscript.

Acknowledgements

Not applicable.

Author details

[1] Department of General Surgery, Shenzhen Baoan District Central Hospital/The Fifth Affiliated Hospital of Shenzhen University, Shenzhen, Guangdong 518000, China.

Abbreviations

KD: ketogenic diets; H&E: hematoxylin and eosin; PBS: phosphate buffer saline; RT-qPCR: real-time quantitative PCR; LCTD: long-chain triacylglycerol diet; MCTD: medium-chain triacylglycerol diet; MAD: modified Atkins diet; LGID: low glycemic index diet.

References

1. Chau I, Cunningham D. Adjuvant therapy in colon cancer–what, when and how? Ann Oncol. 2006;17:1347–59.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.
3. Medical Administration of the National Health and Family Planning Commission of the People's Republic of China CMAOB. China's colorectal cancer diagnosis and treatment specifications (2017 version). Chinese Journal of Practical Surgery. 2018;38:1089–103.
4. Rongshou Zheng KS, Zhang S, Zeng H, Zou X, Chen Ru, Gu X. Wenqiang Wei, Jie He: Analysis of prevalence of malignant tumors in China in 2015. *Chinese Journal of Oncology* 2019:19–28.
5. O'Keefe SJ. Diet, microorganisms and their metabolites, and colon cancer. Nat Rev Gastroenterol Hepatol. 2016;13:691–706.
6. Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer. Gastroenterology. 2010;138:2044–58.
7. Schirrmacher V. From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review). Int J Oncol. 2019;54:407–19.
8. Goodman M. Managing the Side Effects of Chemotherapy. Semin Oncol Nurs. 1989;5:29–52.
9. Maurizio E, Joerg K, Baerbel L, Hans H. Ketogenic Diets in the Treatment of Epilepsy. Curr Pharm Des. 2017;23:5691–701.
10. Ulamek-Koziol M, Czuczwar SJ, Januszewski S, Pluta R. Ketogenic Diet and Epilepsy. *Nutrients* 2019, 11.
11. Paoli A. Ketogenic diet for obesity: friend or foe? Int J Environ Res Public Health. 2014;11:2092–107.
12. Clemens Z, Tóth C. Successful treatment of a patient with obesity, type 2 diabetes and hypertension with the paleolithic ketogenic diet. International Journal of Case Reports Images. 2015;6:161.
13. Bose S, Le A. Glucose Metabolism in Cancer. Adv Exp Med Biol. 2018;1063:3–12.
14. Hagihara K, Kajimoto K, Osaga S, Nagai N, Shimosegawa E, Nakata H, Saito H, Nakano M, Takeuchi M, Kanki H, et al: Promising Effect of a New Ketogenic Diet Regimen in Patients with Advanced Cancer. *Nutrients* 2020, 12.
15. Weber DD, Aminzadeh-Gohari S, Tulipan J, Catalano L, Feichtinger RG, Kofler B. Ketogenic diet in the treatment of cancer - Where do we stand? Mol Metab. 2020;33:102–21.
16. Daniela D, Weber SA-G. Barbara Kofler: Ketogenic diet in cancer therapy *Aging* 2018, 10:164–165.
17. Matteo Vergati EK, Girolamo D, Monte S, Riondino D, Vallone F, Guadagni. Patrizia Ferroni, Mario Roselli: Ketogenic Diet and Other Dietary Intervention Strategies in the Treatment of Cancer. Curr Med Chem. 2017;24:1170–85.
18. Smyl C. Ketogenic Diet and Cancer-a Perspective. Recent Results Cancer Res. 2016;207:233–40.

19. Klement RJ. Fasting, Fats, and Physics: Combining Ketogenic and Radiation Therapy against Cancer. Complement Med Res. 2018;25:102–13.
20. Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, D'Agostino D, Planavsky N, Lupfer C, Kanneganti TD, et al. The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. Nat Med. 2015;21:263–9.
21. Klement RJ. The emerging role of ketogenic diets in cancer treatment. Curr Opin Clin Nutr Metab Care. 2019;22:129–34.
22. Boison D. New insights into the mechanisms of the ketogenic diet. Curr Opin Neurol. 2017;30:187–92.
23. Hao GW, Chen YS, He DM, Wang HY, Wu GH, Zhang B. Growth of human colon cancer cells in nude mice is delayed by ketogenic diet with or without omega-3 fatty acids and medium-chain triglycerides. Asian Pac J Cancer Prev. 2015;16:2061–8.
24. Cury FL, Bhindi B, Rocha J, Scarlata E, El Jurdi K, Ladouceur M, Beauregard S, Vijh AK, Taguchi Y, Chevalier S. Electrochemical red-ox therapy of prostate cancer in nude mice. Bioelectrochemistry. 2015;104:1–9.
25. Yang H, Lee HJ, Lee YG, Kim JW, Kim YS. Integrity of the Untorn Articular-Sided Tendon in Bursal-Sided Partial-Thickness Rotator Cuff Tear: A Comparative Study of Apoptotic Activity in Torn and Untorn Layers. Am J Sports Med. 2018;46:2478–85.
26. Gao ZH, Deng CJ, Xie YY, Guo XL, Wang QQ, Liu LZ, Lee WH, Li SA, Zhang Y. Pore-forming toxin-like protein complex expressed by frog promotes tissue repair. FASEB J. 2019;33:782–95.
27. Guo XL, Liu LZ, Wang QQ, Liang JY, Lee WH, Xiang Y, Li SA, Zhang Y. Endogenous pore-forming protein complex targets acidic glycosphingolipids in lipid rafts to initiate endolysosome regulation. Commun Biol. 2019;2:59.
28. Yujia Liu YZ. Like Zhong,Ping Huang: Research Progress on Effect of Tumor Environment in Tumor Metabolic. Journal of Chinese Oncology. 2020;26:47–52.
29. Ferreira LM. Cancer metabolism: the Warburg effect today. Exp Mol Pathol. 2010;89:372–80.
30. Ivan L. Cameron VAO. Parenteral Level of Glucose Intake on Glucose Homeostasis, Tumor Growth, Gluconeogenesis, and Body Composition in Normal and Tumor-bearing Rats. Can Res. 1983;43:5228–34.
31. Li Z, Heber D. Ketogenic Diets. JAMA. 2020;323:386–6.
32. Martin-McGill KJ, Jackson CF, Bresnahan R, Levy RG, Cooper PN. Ketogenic diets for drug-resistant epilepsy. Cochrane Database Syst Rev. 2018;11:CD001903.
33. Yancy WS Jr, Mitchell NS, Westman EC. Ketogenic Diet for Obesity and Diabetes. JAMA Internal Medicine. 2019;179:1734–5.
34. Allen BG, Bhatia SK, Anderson CM, Eichenberger-Gilmore JM, Sibenaller ZA, Mapuskar KA, Schoenfeld JD, Buatti JM, Spitz DR, Fath MA. Ketogenic diets as an adjuvant cancer therapy: History and potential mechanism. Redox Biol. 2014;2:963–70.

35. Wang Q, Zhou Y, Rychahou P, Fan TW, Lane AN, Weiss HL, Evers BM. Ketogenesis contributes to intestinal cell differentiation. Cell Death Differ. 2017;24:458–68.
36. Zhou Y, Rychahou P, Wang Q, Weiss HL, Evers BM. TSC2/mTORC1 signaling controls Paneth and goblet cell differentiation in the intestinal epithelium. Cell Death Dis. 2015;6:e1631.
37. Nusse R. Wnt signaling and stem cell control. Cell Res. 2008;18:523–7.
38. Ahmadzadeh A, Norozi F, Shahrabi S, Shahjahani M, Saki N. Wnt/beta-catenin signaling in bone marrow niche. Cell Tissue Res. 2016;363:321–35.
39. Wang W, Smits R, Hao H, He C. Wnt/beta-Catenin Signaling in Liver Cancers. *Cancers (Basel)* 2019, 11.
40. Martin-Orozco E, Sanchez-Fernandez A, Ortiz-Parra I, Ayala-San Nicolas M. WNT Signaling in Tumors: The Way to Evade Drugs and Immunity. Front Immunol. 2019;10:2854.
41. Prossomariti A, Piazzi G, Alquati C, Ricciardiello L. Are Wnt/beta-Catenin and PI3K/AKT/mTORC1 Distinct Pathways in Colorectal Cancer? *Cell Mol Gastroenterol Hepatol* 2020.
42. Kim JY, Park G, Krishnan M, Ha E, Chun K-S. Selective Wnt/β-catenin Small-molecule Inhibitor CWP232228 Impairs Tumor Growth of Colon Cancer. Anticancer Res. 2019;39:3661–7.
43. Flores-Hernandez E, Velazquez DM, Castaneda-Patlan MC, Fuentes-Garcia G, Fonseca-Camarillo G, Yamamoto-Furusho JK, Romero-Avila MT, Garcia-Sainz JA, Robles-Flores M. Canonical and non-canonical Wnt signaling are simultaneously activated by Wnts in colon cancer cells. Cell Signal. 2020;72:109636.
44. Cheng X, Xu X, Chen D, Zhao F, Wang W. Therapeutic potential of targeting the Wnt/beta-catenin signaling pathway in colorectal cancer. Biomed Pharmacother. 2019;110:473–81.

Table

Table 1 is not provided with this version of the manuscript.

Figures

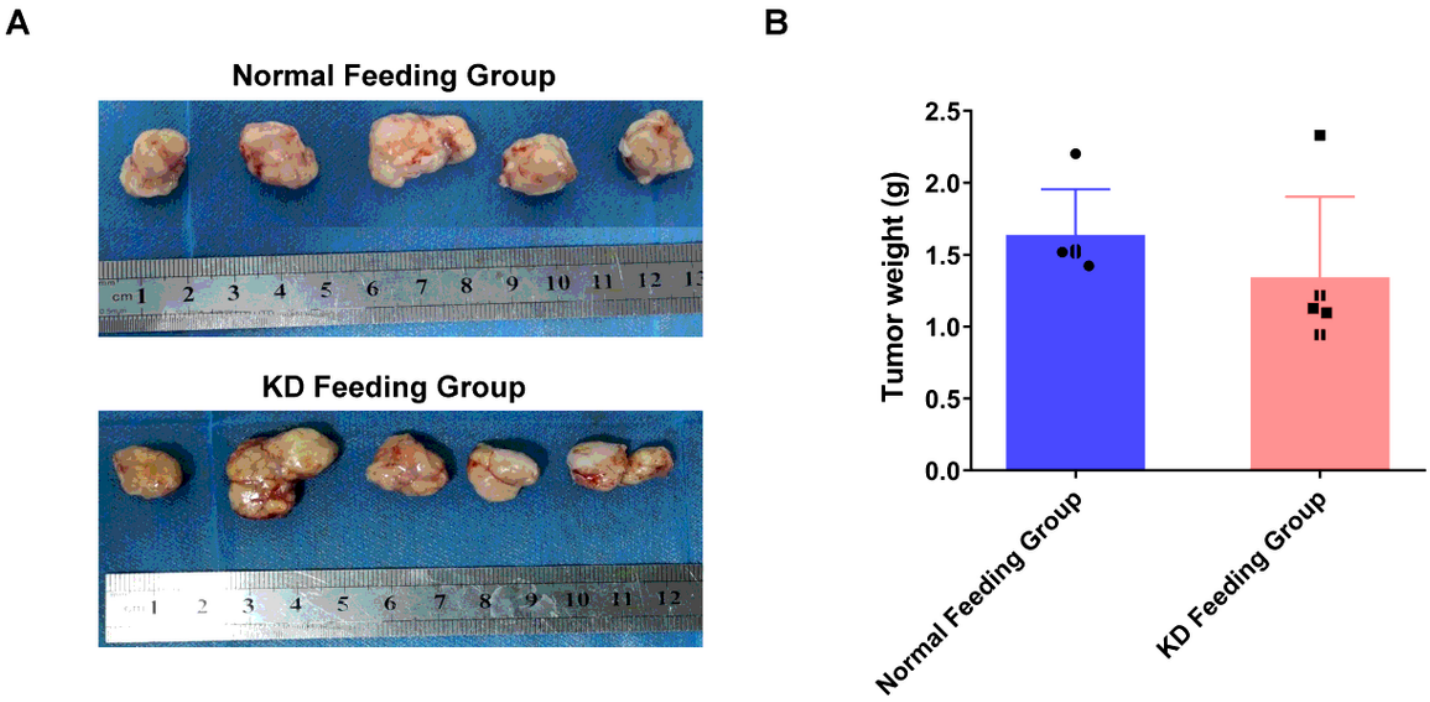

Figure 1

The size of subcutaneous tumor in nude mouse was not affected by KD feeding. (A) The subcutaneous tumors were prepared and their size were measured after the xenograft nude mice fed with KD or normal diet 30 days (n = 5). The tumor mass were representative of three independent experiments. (B) The weights of subcutaneous tumors were measured after the xenograft nude mice fed with KD or normal diet 30 days (n = 5). There was no significant difference between normal feeding group and KD feeding group by using unpaired two-tailed Student's t test (n = 5).

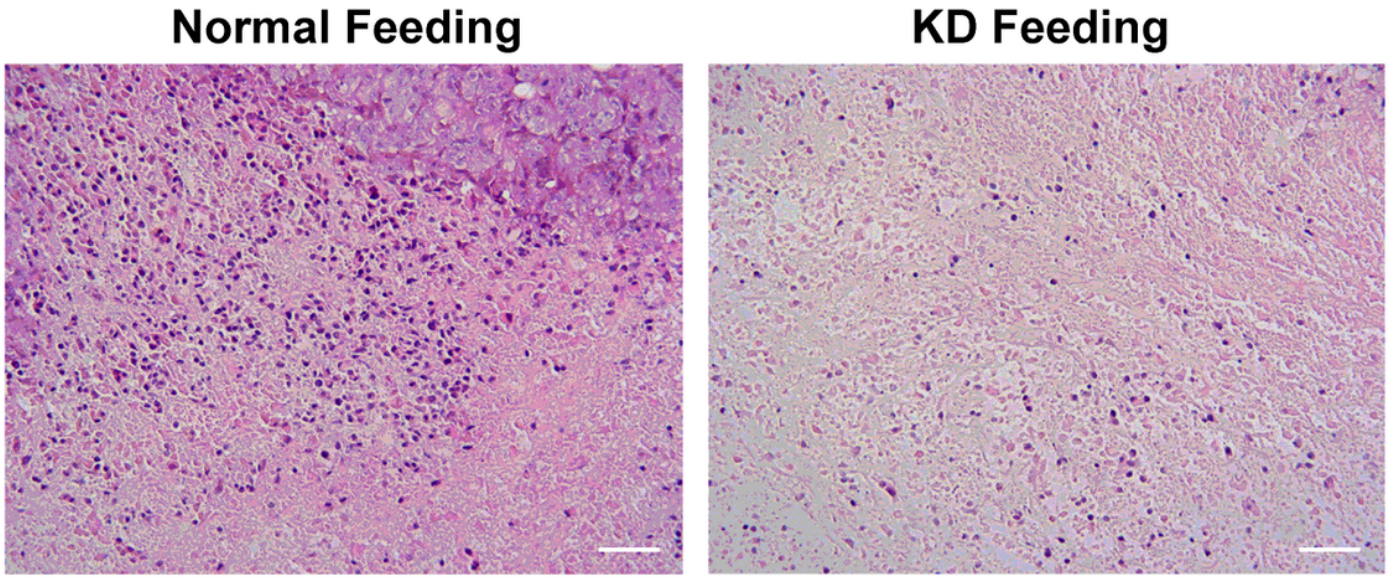

Figure 2

H&E staining of subcutaneous tumor in nude mouse xenograft models. The subcutaneous tumors were prepared and stained with hematoxylin and eosin after the xenograft nude mice fed with KD or normal diet 30 days. The histopathological characteristics and inflammatory infiltration of tumor were observed under light microscopy. Scale bar = 100 μm.

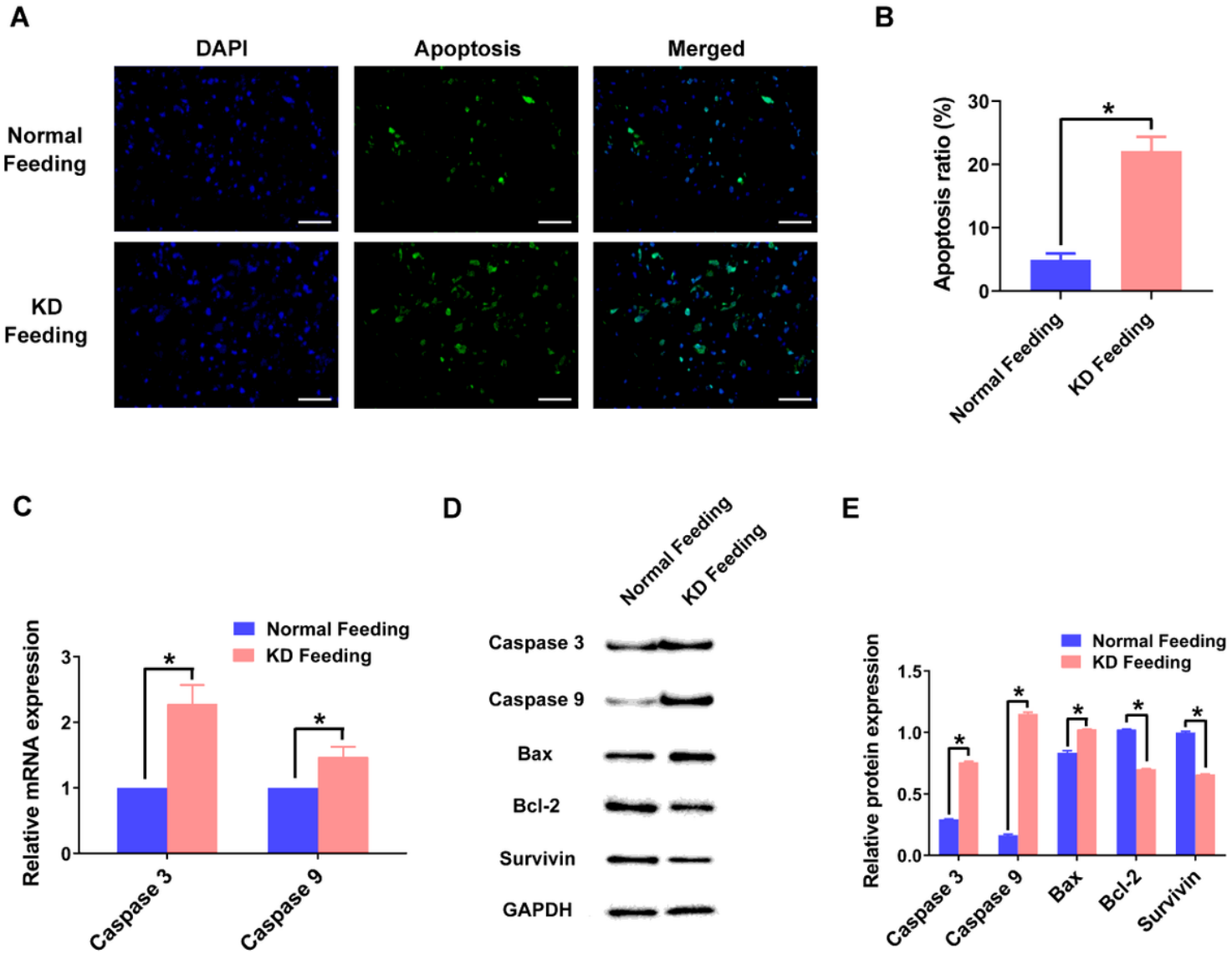

Figure 3

KD promoted the apoptosis of of subcutaneous tumor in xenograft nude mouse models. (A) After the xenograft nude mice fed with KD or normal diet 30 days, the subcutaneous tumors were obtained and the apoptosis of tumor cells were determined by TUNEL staining and observed under confocal microscopy. Scale bar = 100 μm. (B) The apoptosis ratio of subcutaneous tumors was quantified by analyzing the fluorescence intensity of apoptotic cells from 5 individual views. *P < 0.05 versus normal feeding group by using unpaired two-tailed Student's t test (n = 5). (C) The expressions of caspase 3 and caspase 9 mRNA in subcutaneous tumors were detected by qRT-PCR after the xenograft nude mice fed with KD or normal diet 30 days. The relative mRNA expressions in the samples were normalized against the expression of GAPDH, and the mRNA expression in normal feeding group was normalized to 1. Bars

represent the mean ± SD from three independent experiments. *P < 0.05 versus control by using one-way ANOVA with Bonferroni correction (n = 5). (D) The expressions of caspase 3, caspase 9, Bax, Bcl-2 and survivin proteins in subcutaneous tumors were detected by western blot after the xenograft nude mice fed with KD or normal diet 30 days. The immunoblots are representative of three independent experiments. (E) The band intensities of caspase 3, caspase 9, Bax, Bcl-2 and survivin proteins were quantitative analyzed according to their gray by Image J software. Bars represent the mean ± SD from three independent experiments. *P < 0.05 versus control by using one-way ANOVA with Bonferroni correction (n = 5).

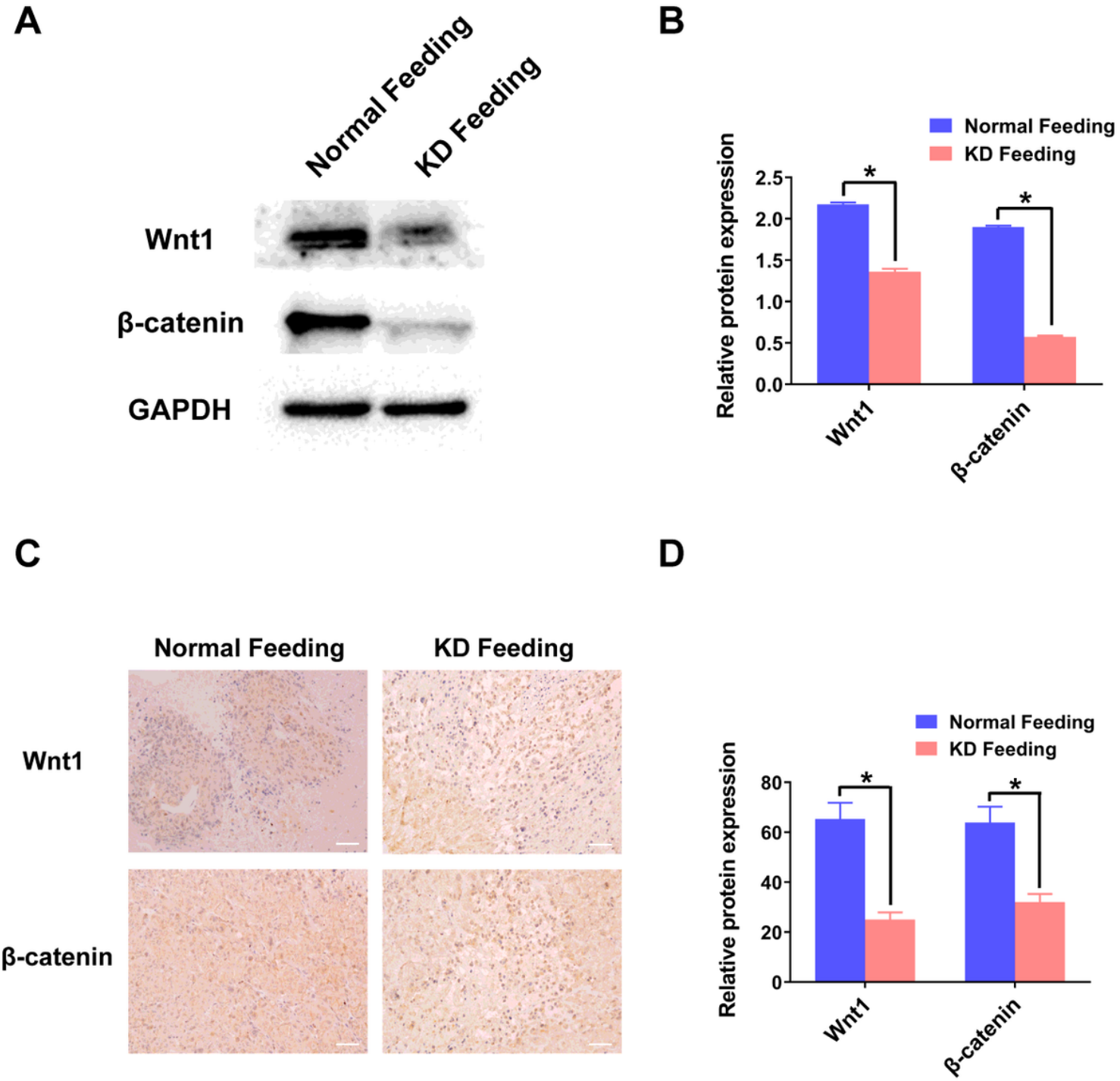

Figure 4

KD inhibited the expression of Wnt1/β-catenin signaling proteins. (A) After the xenograft nude mice fed with KD or normal diet 30 days, the expression of Wnt1 and β-catenin proteins of subcutaneous tumors were determined by western blot. The immunoblots are representative of three independent experiments. (B) The band intensities of Wnt1 and β-catenin proteins were quantitative analyzed according to their gray by Image J software. (C) After the xenograft nude mice fed with KD or normal diet 30 days, the subcutaneous tumors were obtained and the expression of Wnt1 and β-catenin proteins were determined by immunohistochemistry and observed under light microscopy. Scale bar = 100 μm. (D) The expressions of Wnt1 and β-catenin proteins were quantified by analyzing the brown colored intensity from 5 individual views. Bars represent the mean ± SD from three independent experiments. *P < 0.05 versus normal feeding group by using one-way ANOVA with Bonferroni correction (n = 5).