Colorectal cancer cells with stably expressed SIRT3 demonstrate proliferating retardation by Wnt/β-catenin cascade inactivation

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Abstract

Colorectal cancer (CRC) is a typical and lethal digestive system malignancy. In this study, we investigated the effect of Sirtuin 3 (SIRT3) expression, a fidelity mitochondrial protein, on proliferation of CRC cells and the related mechanisms. Using UALCAN database and CPTAC database, we found low expression of SIRT3 in CRC was the unfavorable factor for survival prognosis. Meanwhile,SIRT3 expression was correlated with distant metastasis and TNM stage of CRC patients. Subsequently, we found the proliferating capacities of CRC cells with stably expressed *SIRT3* were decreased dramatically *in vitro* and *in vivo*, with comparison to their counterparts, respectively. Further western blot (WB), immunoprecipitation (IP) and TOPflash/FOPflash assay showed the related mechanism of growth retardation of these cells was highly associated with the degradation of β-catenin in cytosol, and the localization of β-catenin/α-catenin complex in nucleus. Taken together, these results revealed the retardation of CRC cell proliferation by SIRT3 was highly correlated with the inactivation of Wnt/β-catenin signaling.

TITLE: Colorectal cancer cells with stably expressed *SIRT3* demonstrate proliferating retardation by Wnt/β-catenin cascade inactivation

ABBREVIATED TITLE : SIRT3 suppresses CRC growth by Wnt/β-catenin

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Abstract

Colorectal cancer (CRC) is a typical and lethal digestive system malignancy. In this study, we investigated the effect of Sirtuin 3 (SIRT3) expression, a fidelity mitochondrial protein, on proliferation of CRC cells and the related mechanisms. Using UALCAN database and CPTAC database, we found low expression of SIRT3 in CRC was the unfavorable factor for survival prognosis. Meanwhile,SIRT3 expression was correlated with distant metastasis and TNM stage of CRC patients. Subsequently, we found the proliferating capacities of CRC cells with stably expressed *SIRT3* were decreased dramatically *in vitro* and *in vivo* , with comparison to their counterparts, respectively. Further western blot (WB), immunoprecipitation (IP) and TOPflash/FOPflash assay showed the related mechanism of growth retardation of these cells was highly associated with the degradation of β-catenin in cytosol, and the localization of β-catenin/α-catenin complex in nucleus. Taken together, these results revealed the retardation of CRC cell proliferation by SIRT3 was highly correlated with the inactivation of Wnt/β-catenin signaling.

KEY WORDS

Colorectal cancer, Proliferation, *SIRT3* , Wnt/β-catenin cascade

ABBREVIATION LIST

Sirt3: Sirtuin 3

UALCAN: The University of Alabama at Birmingham Cancer Data Analysis Portal

CPTAC: The Clinical Proteomic Tumor Analysis Consortium

CRC: Colorectal cancer

WB: Western blot

ANOVA: analysis of variance

ROC: receiver operating characteristic

IP: Immunoprecipitation

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

p-β-catenin: Phosphate-β-catenin (Ser 33/37/Thr 47)

INTRODUCTION

Colorectal cancer is a typical and lethal digestive system malignancy that represents the second leading cause of cancer-related death worldwide. Its incidence is increasing rapidly over years, acquiring prevalent social relevance[1]. Comprehensive approaches in CRC treatment including surgery, chemotherapy, immunotherapy, and radiation, were general applied but with limitations, adverse effects, and a high recurrence rate[2]. Therefore, delineating the key regulators participating in CRC growth and metastasis becomes pivotal to support the development of combination therapy strategies.

The progression of CRC is promoted by inflammation, oxidative stress, metastasis, and angiogenesis[3]. More than 90% of CRC patients carry gene mutations that deregulate the Wnt/β-catenin signaling cascade, resulting in the nuclear importing of β-catenin to bind to the T-cell factor/lymphoid enhancer factor 1 (TCF/LEF) transcription factors for transcriptional activation of a cluster of target genes in growth, angiogenesis, metastasis etc[4]. Though it is very clear for the significance between Wnt/β-Catenin signaling and CRC, targeting such an important signaling pathway without clinical side effects remains infeasible due to numerous questions about its regulation.

CRC is characterized by enhanced glycolysis and repressed mitochondrial respiration and dynamics[5]. As one of fidelity mitochondrial proteins, SIRT3 finely regulate the cellular energetic homeostasis and oxidative stress response. As the defender for intestinal function, SIRT3-deficient mice showed hyper-susceptibility to colon inflammation and CRC development[6]. A deluge of literature has elucidated the role of SIRT3 in the progression of CRC with paradoxical results. the anti-tumor effects of 5-fluorouracil[7], glycyrrhizic acid[8] and oxaliplatin[9]were validated through the suppression of SIRT3 in CRC cells. In contrast, the anti-tumor effects of metformin[10], ganoderic acid D[11], ergothioneine[12], δ-valerobetaine[13] were proved by the upregulation of SIRT3 in CRC cells. In the present study, we first analyzed the expression of SIRT3 in CRC with comparison to their matched normal tissues by UALCAN database. CPTAC database was used to analyze the relationship of SIRT3 expression with the overall survival rate as well as clinicopathological parameters of CRC cases. Next, the*SIRT3* stably expressed CRC cell lines, namely HT-29S3 and HCT-116S3, were established by lentiviral transfection. Furthermore, the proliferating capacity of these cell lines was monitored both *in vitro* and *in vivo* , with comparison to their parent cells. Finally, the activation of Wnt/β-catenin signaling pathway was also explored by TOPflasn/FOPflash assay in these cells. Our understanding of the role of SIRT3 in the regulation of Wnt/β-catenin cascade in CRC highlights the potential of SIRT3 as a promising therapeutic target against the progression of CRC.

Results

SIRT3 expression was decreased in CRC tissues and correlated to the prognosis of CRC

UALCAN database showed the significant decrease in SIRT3 expression in CRC tissues compared to their matched normal tissues (Fig. 1A). The decreasing of SIRT3 in CRC was occurred from stage 2 to 4, suggesting a correlation between SIRT3 and tumor histological stages (Fig. 1B). To identify this assumption, SIRT3 protein level of 60 cases in total from CPTAC database with clinical pathological features were downloaded for further analysis. The SIRT3 protein level in CRC patients was quantified by SIRT3 spectral counts, with the cut-off value of 0.5 for all samples (sensitivity, 31.1% ; specificity, 93.3%; P = 0.149). The overall survival ratio of CRC patients was 75.0%. 45 cases were classified into low SIRT3 expression group, with the survival ratio of 54.8%. Comparatively, 15 cases were categorized into high SIRT3 expression group, with the survival ratio of 93.3%. The overall survival of CRC patients was negatively correlated to the expression of SIRT3 with significance (χ^2=5.986, p=0.014), and confirmed by Kaplan-Meier analysis (Fig.1C). Additionally, statistically significant correlation was observed between SIRT3 expression and distant metastasis or TNM stages, respectively. Eventually, the decrease of SIRT3 in CRC tissues was associated with the dysregulation of Wnt pathway as indicated in Fig.1D.

The stable expression of *SIRT3* in CRC cells led to the retardation of cell proliferation *in*

vitro* and *in vivo

To prove the role of SIRT3 in the progress of CRC, the *SIRT3*stable expressed cell lines, namely HT29-S3 and HCT116-S3, were established and validated for the overexpression of SIRT3 protein by WB and SIRT3 enzyme activity measurements. As shown in Fig.2A, the expression levels of SIRT3 in HT29-S3 and HCT116-S3 cells were dramatically increased upon WB measurement in comparison with their parent cells. As the result, the mitochondrial activity of SIRT3 in HT29-S3 and HCT116-S3 was 3.67 and 1.58 times of that in their parent cells, individually (Fig.2B). Proliferation of HT29 and HCT116 was notably reduced after *SIRT3*stably expressed, as indicated by Fig.2C and 2D. Similar results were obtained when these cells were implemented subcutaneously in nude mice (Fig. 2E-2G), which showing the retardation of tumor growth in terms of tumor weight and volume loss in cells with *SIRT3* stably expressed. Notably, the dramatic elevation in SIRT3 expression was observed in HT29-S3 and HCT116-S3 tumor tissues (Fig.2H and 2I), compared to the mild expression of SIRT3 in HT29 and HCT116 tissues.

Τηε ινηιβιτιον οφ τηε προλιφερατιον οφ *ΣIPT3* σταβλψ εξπρεσσεδ ςελλς ωας ηιγηλψ ςορρελατεδ ωιτη τηε συππρεσσιον οφΩντ/β-ςατενιν ςασςαδε

As the central molecule and crucial nuclear effector, the membrane localization, post-transcriptional modification in cytosol for its degradation, and coactivator/corepressor of β-catenin in nucleus plays pivotal role in the activation of Wnt/β-catenin cascade[13,14]. Unexpectedly, the expression of β-catenin in cell membrane decreased significantly in HT29-S3 cells, compared to HT29 cells (Fig.3A). However, the expression of p120-catenin was significantly increased in the membrane of HT29-S3 cells, indicating the role of E-cadherin/p120-catenin complex but not the E-cadherin/β-catenin complex in the adherence of HT9-S3 cells. Furthermore, the p-β-catenin in cytosol, the degradation form of β-catenin, was increased in HT29-S3 cells (Fig.3B), indicating the promotion of β-catenin degradation by SIRT3. Moreover, the expression of β-catenin in nucleus was also elevated in HT29-S3 cells compared to that in HT29 cells (Fig.3C). For the identification of the effect of SIRT3 in regulating gene transcription by nuclear β-catenin, TOPflash/FOPflash assay was conducted in these cells. As pointed out in Fig.3D, the luciferase activity of TOPflash plasmid was remarkably reduced in HT29-S3 cells(Fig.3E).

To comprehensively explored the incompatible observation between the elevated nuclear β-catenin concentration and suppressed TOPflash luciferase activity, nuclear proteins from cells were subjected to IP assay to determine the corepressors in β-catenin complex for gene transcriptional regulation. As expected, the interaction between α-E-Catenin and β-catenin was surprisingly increased in the nucleus of HT29-S3 cells than that in HT29 cells (Fig.3D).

Discussion

Despite the advances in prognostic and therapeutic approach in CRC, metastasis, chemoresistance, recurrence of CRC are still the focus and dilemma of clinical treatments. The hyperactivation of Wnt/β-catenin signaling plays pivotal role in cancer-related processes, such as cancer stem cell propagation, angiogenesis, epithelial-mesenchymal transition, chemoresistance and metastasis. As a scaffold protein and the effector of Wnt/β-catenin signaling pathway, β-catenin activity is not only related to its level in the nucleus, but also depends on its coregulators to activate or suppress downstream gene transcription[15]. These positive or negative regulators for β-catenin activity still needs further investigation to explore the potential regulatory mechanism of β-catenin-dependent transcription, and thereby facilitating the development of novel therapies targeting the Wnt/β-catenin pathway.

In this study, the inhibitory role of SIRT3 on CRC growth was investigated and confirmed to be associated with the Wnt/β-catenin signaling inactivation. This result is consistent with the analytical results from UALCAN database, which not only showed that SIRT3 expression decreased in cancer tissues with the progression of CRC, but also was associated with the alteration of Wnt /β-catenin signaling pathway. This led us to construct *SIRT3* stably expressed cell lines to imitate the persistence of SIRT3 expression *in vivo* and confirm the above assumption. The experimental results clearly indicated the negative correlation between SIRT3 level and tumor growth, as well as the activation of Wnt /β-catenin cascade. The inactivation of

Wnt /β-catenin cascade was also observed in HT29 cells with *SIRT3*knockout by Cas9/CRISPR technology, but it was not as dramatic as in HT29-S3 cells. Further study has revealed that the cell migration capacity was increased in HT29 cells without *SIRT3* expression by the scratching experiment, with comparison to HT29-S3 cells (data not shown).

SIRT3 promoted the binding of α-E-Catenin to β-catenin in the nucleus of colorectal cancer cells, resulting in the inhibition of β-catenin activity. To the best of our knowledge, this is the first observation in the regulation of β-catenin activity by SIRT3 via α-E-Catenin in CRC cells. α-E-Catenin is universally expressed in normal human tissues, participating in building and maintaining actin organization. Accumulated evidences have demonstrated its role as the tumor suppressor in CRC progress by inhibiting the Wnt/β-catenin pathway[16,17], particularly in the regulation of β-catenin level and activity. However, we failed to detect the direct interaction between SIRT3 and β-catenin or α-E-Catenin. Therefore, it would be of great interest to further explore how SIRT3 in regulating β-catenin by α-E-Catenin.

Taken together, we herein identified the tumor suppressor role of SIRT3 in the progress of CRC by inactivation of Wnt/β-catenin pathway, through directly promoting the interaction of β-catenin to α-E-Catenin. This finding will lay a theoretical and experimental basis for the underlying mechanism of β-catenin regulation in CRC, thereby highlight the potential role of SIRT3 against tumor progression and recurrence.

MATERIALS AND METHODS

Bioinformatic statistics of SIRT3 expression in UALCAN and CPTAC

The expression of SIRT3 between CRC and normal colon tissues was analyzed in the UALCAN database (http://ualcan.path.uab.edu/index.html). The protein mass spectrometry of 60 cases in CPTAC database (https://pdc.cancer.gov/) were extracted with clinical data for retrospectively analysis regarding to the correlation of SIRT3 expression with the overall survival rate, and clinical pathological characteristics. Detailed information on these cases is in Table 1 and Figure 1.

Generation of SIRT3 stably expressed CRC cell lines

Human CRC cell lines, HT29 and HCT116, were maintained in McCoy's 5A medium (Servicebio, China) supplemented with 10% FBS (GIBCO, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin. The cells lines above were grown at 37 °C incubators in a humidified 5% CO_2 atmosphere. To generate *SIRT3*stably expressed CRC cells, these cells were planted into a 6-well plate at a density of 5×10^5/well. After 24 h, cells were incubated with the medium containing lentivirus, pLV[Exp]-Puro-CMV>hSIRT3[NM_012239.6] (Vectorbuilder, USA), following the manufacture's instruction. After incubating in 37 for 24 h, culture medium containing lentivirus was replaced with the fresh medium containing 2 μg/ml Puromycin for 14 days. The expression and activity of obtained cells were verified by WB and SIRT3 activity measurement.

SIRT3 activity measurement

Mitochondria was isolated from cells using the kit following the manufacturer's instruction (Invent, USA). SIRT3 activity of the mitochondrial protein from each cell line was monitored by SIRT3 direct fluorescent screening assay kit from Cayman Chemical Co. by following the manufacture's instruction.

Proliferation assay

Cell proliferation was detected by MTT as described elsewhere. Briefly, cells were planted in a 96-well plate at the density of 1×10^3/well, and the cell viability was determined by a multi-functional microwell plate detector via reading the optical density at 490 nm in each well after planting for 48, 96, and 144 hrs.

TOPFLASH and FOPFLASH assay

1 μg TCF/LEF luciferase reporter plasmid (TOPflash) (D2501, Beyotime, China) or 1 μg mutated TCF/LEF luciferase reporter (FOPflash) (D2503, Beyotime, China) plasmid was used to co-transfect CRC cells with 0.05 μg Renilla-expressing vector by X-tremeGene HP DNA transfection reagent (Merck, USA) according to the manufacturer's protocol, respectively. Firefly and Renilla luciferase activities were measured in cell

lysate 48 h after transfection using the Dual-Lumi II Luciferase reporter gene assay kit (Beyotime, China). The Renilla luciferase units were used as the control to calculate the relative activities of the promoter. The ratio of TOPflash to FOPflash luciferase activity was considered as the relative activity of the promoter.

Cell lysis, IP and WB

Proteins from cell membrane, cytosol and nuclei were isolated by commercial kits following manufacturer's introduction (Invent, USA). Proteins from cell membrane and cytosol were further subjected to WB with antibodies including SIRT3 (2627S, CST, USA), Na,K-ATPase (23565S, CST, USA), β-Catenin (8480S, CST, USA), Phospho-β-Catenin (Ser33/37/Thr41) (9561S, CST, USA), E-Cadherin (3195S, CST, USA), Histone-H3 (4499S, CST, USA), p120-catenin (66208-1-Ig, ProteinTech, USA), α-E-Catenin (3240S, CST, USA), and GAPDH (60004-1-Ig, ProteinTech, USA). For immunoprecipitation, the nuclear extracts (1 mg in total) were precleaned by rotation for 1 h with 50 μl of Dynabeads protein G magnetic beads (10007D, ThermoFisher Sci, USA), then subjected to the incubation with magnetic beads pre-binding with the antibody of choice. After overnight incubation at 4, the beads were washed and pellets were collected as immunoprecipitated complexes, and further subjected to WB as described elsewhere. Each protein of interest was analyzed in samples for at least three independent experiments from each set of tumor cells. Images were recorded using a luminescent image analyzer, and the intensities of the bands were quantitated by densitometry (NIH Image J software).

In vivo **tumorigenicity Assay**

Six-week-old male BALB/c nude mice were provided by Beijing Vital River Laboratory Animal Technology Company. To establish a xenograft model, the mice were subcutaneously injected with 2×10^6 cells that w/o stably transfected with *Sirt3* . The volume of xenografts was monitored every two days. All mice were sacrificed when the volume of xenografts reached to 2000 mm^3, and then the xenografts were removed, weighed, and photographed. All animal experiments were approved by the ethics committee of Capital Medical University.

Statistical method

SPSS (v26.0; IBM Corp.,NY, USA) were used for statistical analysis and data generation. The cut-off values for SIRT3 expression in CRC patients were obtained using receiver operating characteristic (ROC) curve evaluation. Chi-square test was applied to analyze the correlation between SIRT3 expression and clinical characteristics. Survival analysis was performed by Kaplan-Meier method. The COX proportional hazards regression models were used for univariate analysis. The significant differences were analyzed using the Student t test (between the 2 groups) or one-way analysis of variance (multiple groups). A p-value less than 0.05 was considered statistically significant.

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Tables

Table 1. *Cox proportional Hazard regression analysis of patients' overall survival based on data from CPTAC database stratified by expression of SIRT3 protein status*

Clinicopathologic indexes	Survival	Survival	χ^2	P value	SIRT3 protein	SIRT3 protein	χ^2	P value
	DEAD	Alive			Low	High		
Gender							0.659	0.417
Male	21	9	0.800	0.371	24	6		
Female	24	6			21	9		
Age in diagnosis(years)			0.769	0.380			0.988	0.368?¿?
60	7	1			7	1		
>60	38	14			38	14		
Location			1.604	0.205			0.715	0.379
Colon	37	10			37	10		
Cecum	8	5			8	5		
Distant metastasis			6.173	0.013*			5.365	0.021*
No	43	11			40	14		

Clinicopathologic indexes	Survival	Survival	χ^2	P value	SIRT3 protein	SIRT3 protein	χ^2	P value
Yes	2	4			5	1		
Lymph node metastasis			3.361	0.186			2.142	0.307
0	28	6			25	9		
1	9	3			18	5		
2	8	6			2	1		
TNM stages			4.000	0.046*			5.798	0.016*
T1+T2	10	0			5	5		
T3+T4	35	15			40	10		
Tumor stages			4.434	0.035*			1.545	0.211
T1+T2	29	5			25	9		
T3+T4	16	10			20	6		
SIRT3 protein level			5.986	0.014*				
Low	14	31						
High	1	14						

Figures

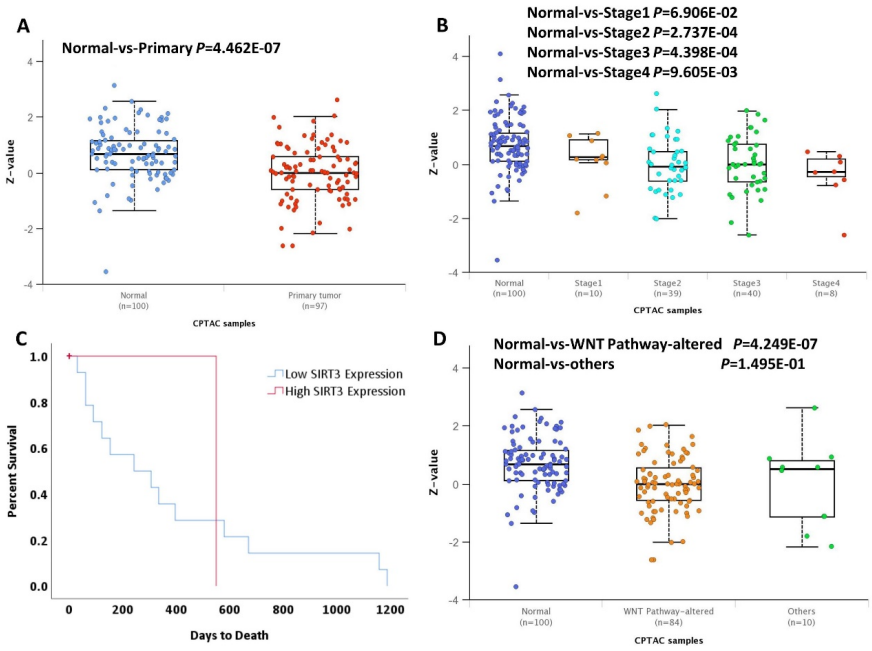

Fig. 1. *Predict the role of SIRT3 in CRC based on bioinformatics analysis. (A) The expression of SIRT3 in CRC tissues is significantly lower than that of matched normal tissues. (B) The expression of SIRT3 in normal tissues and CRC tissues of different stage. (C) Kaplan-Meier plot of the overall survival of patients stratified by SIRT3 expression was analyzed based on data from CPTAC database. (D) The decrease of SIRT3 in CRC tissues is associated with the dysregulation of Wnt pathway.*

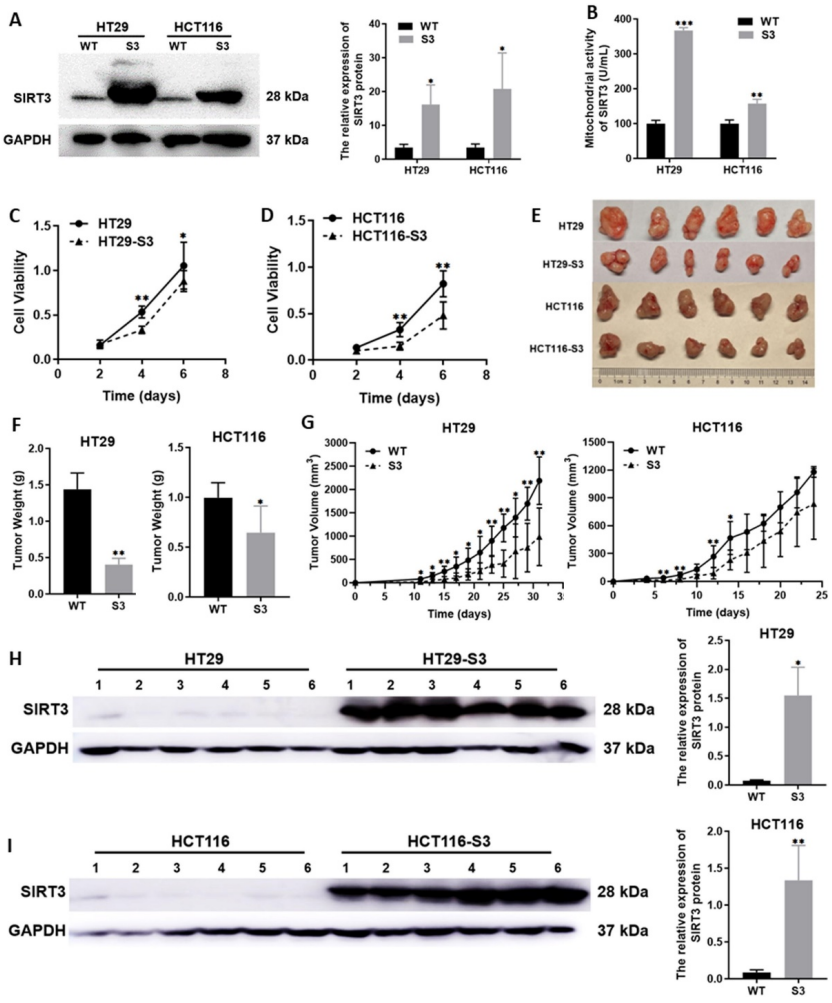

Fig. 2. *The effect of SIRT3 on the proliferation of CRC cells in vitro and in vivo. (A) The expression of SIRT3 protein in HT29 and HCT116 cell lines after lentivirus infection determined by WB. (B)*

*The mitochondrial activity of SIRT3 determined by Kit. (C)& (D) The stable expression of SIRT3 inhibited the proliferation of CRC cells detected by MTT. (E) Tumor xenografts were established by subcutaneous inoculation of CRC cells into the armpit of nude mice. Xenograft weight (g) (F) and size (mm) (G) were measured. (H)&(I) WB assays of the expression of SIRT3 protein from WT/S3 xenograft tumors. *P<0.05, **P<0.01, ***P<0.001*

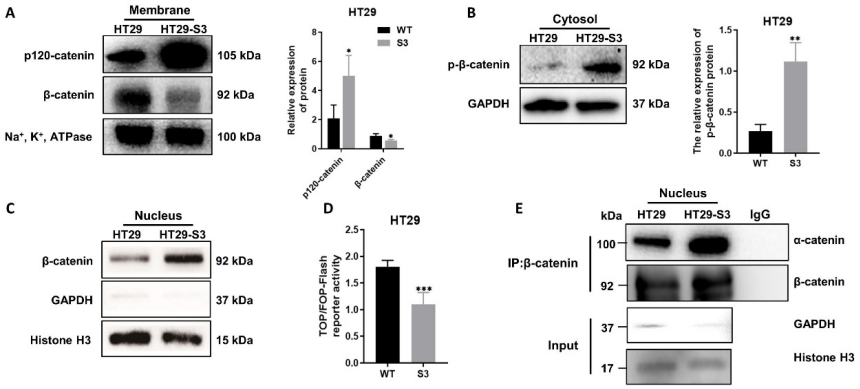

Fig. 3. *Τηε σταβλε εξπρεσσιον οφ ΣΙΡΤ3 συππρεσσεδ Ωντ/β-σατενιν σασαδε. Τηε σταβλε εξπρεσσιον οφ ΣΙΡΤ3 σηνιφισαντλψ δεσρεασεδ τηε εξπρεσσιον οφβ-σατενιν ανδ ινσρεασεδ π120-σατενιν ιν σελλ μεμβρανε (Α), ινσρεασεδ π-β-σατενιν ιν σψτοσολ (Β) ανδ τηε εξπρεσσιον οφ β-σατενιν ιν νυσλευς (Ͳ) δετεστεδ βψ ΩΒ. (Δ) Τηε εφφεστ οφ ΣΙΡΤ3 δετερμινεδ βψ β-σατενιν-ΤͲΦ/ΛΕΦ τρανσσριπτιοναλ αστιτψ υσινγ τηε ΤΟΠφλαση/ΦΟΠφλαση ασσαψ. Λυσιφερασε ρεπορτερ αστιτψ ωας νορμαλιζεδ το Ρενιλλα αστιτψ. (Ε) Ιντεραστιον βετωεεν β-σατενιν ανδ α-σατενιν ωας οβσερεδ βψ ΙΙΙ ιν νυσλευς. *Π<0.05, **Π<0.01, ***Π<0.001*