High levels of SIRT1 expression enhance tumorigenesis and associate with a poor prognosis of colorectal carcinoma patients

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SIRT1, a NAD+ dependent class III deacetylase, takes part in many important biological processes. Previous studies show that SIRT1 is overexpressed in some cancers and plays an essential role in tumorigenesis. However, the association between SIRT1 and colorectal cancer (CRC) is still unclear. We found that many CRC specimens had strong SIRT1 expression, which had an obvious correlation with poor prognosis of CRC patients. Meanwhile, SIRT1 expression had a co-localization with CD133, a current universal marker to characterize colorectal cancer stem cells (CSCs). In vitro studies also revealed that SIRT1 was overexpressed in colorectal CSC-like cells. Moreover, SIRT1 deficiency decreased percentage of CD133+ cells, attenuated the abilities of colony and sphere formation, and inhibited tumorigenicity in vivo in CRC cells. Further study demonstrated that the expressions of several stemness-associated genes, including Oct4, Nanog, Cripto, Tert and Lin28, were reduced by SIRT1 knockdown in CRC cells. Taken together, our findings suggest that SIRT1 plays a crucial role in keeping the characteristics of CSCs cells. SIRT1 is a potential independent prognostic factor of CRC patients after tumor resection with curative intent, and will contribute to providing a promising new approach to target at CSCs in CRC treatment.

Colorectal cancer (CRC) is a kind of cancer causing by uncontrolled cells growth in the colon or rectum. Although CRC has a fully-understood genetic risk, it is still the third most common cancer in the world, with nearly 1.4 million new cases in 2012. In America, CRC is one of the four major cancers, with 9% of cancer deaths. In Europe, the five-year survival rate of CRC is less than 60%, and a third of patients die from it. The principal treatment of CRC is operation combined with postoperative chemotherapy and radiation therapy. However, radical cure for recurrent and metastasis CRC still is a major difficulty. Treatment status indicates that there is a subpopulation of cancer cells, in other words, cancer stem cells (CSCs), which cannot be eradicated by current therapies. CSCs are a rare population of cancer cells which possess the ability of self-renewal and differentiation into multiple cell types. These cells can initiate and sustain tumor growth. Meanwhile, CSCs have strong resistances towards chemotherapeutic agent and radiation therapy. Owing to the ability of tumor formation and maintenance, CSCs are considered to be responsible for the poor prognosis. The CSCs subpopulation of CRC cells was also identified. These CSCs promoted the CRC progression and recurrence. Increasing therapies targeted at CSCs have attracted tremendous attentions in recent years.

SIRT1, the human homolog of Sir2, is a member of sirtuins family. SIRT1 is a NAD+ dependent class III deacetylase (HDAC) which can deacetylate both histone and non-histone proteins. SIRT1 takes part in numerous cellular processes by the deacetylation of specific substrates. Previous evidence suggests that SIRT1 down regulates the activation of p53 as a transcription factor by deacetylating the C-terminal Lys120, Lys164 and Lys382 residues. Moreover, SIRT1 influences cell survival by deacetylating Ku70, Bax and E2F1. SIRT1 also has an impact on senescence, differentiation and oxidative stress resistance. In addition, SIRT1 is considered as an essential role in tumorigenesis. Recent studies demonstrate that SIRT1 is overexpressed in some cancers, such as prostate cancer, breast cancer and leukemia lymphoblasts. However, the function of SIRT1 in tumorigenesis and therapy development remains to be fully understood.
tumor initiation and progression is still under debate. Meanwhile, SIRT1 also plays an essential role in maintaining the self-renewal ability and pluripotency of embryonic stem cells (ESCs). SIRT1 maintains the properties of ESCs by taking part in the Oct4-SIRT1-p53 axis, and/or regulating the expression of Nanog. These studies indicate that SIRT1 may have underlying association with CSCs. In the present work, we firstly detected whether the SIRT1 expression of cancer tissue had associations with prognosis and distribution of CSC-like cells in human CRC patients. Then we further explored the influences of SIRT1 on CSCs in tumorigenesis and their underlying mechanisms in CRC cell lines.

**Results**

**Strong SIRT1 expression of tumor tissues had a correlation with poor prognosis in CRC patients.** Data from a total of 102 patients with colorectal adenocarcinoma were evaluated. Demographics of 102 CRC patients are listed in Table 1. The expression of SIRT1 was detected by immunohistochemical analysis. Data revealed that SIRT1 had a nuclear localization in CRC tissues, and CRC tissue had stronger SIRT1 expression compared with that of corresponding pericarcinomatous tissue (Figure 1A). Then SIRT1 expression was scored according to the SIRT1+ tumour glands. Tumours with more than 50% (including 50%) SIRT1+ glands were defined as SIRT1-Strong, whereas less than 50% stained glands were considered as SIRT1-Weak. Immunohistochemical staining of SIRT1 showed that CRC specimens with weak and strong expression were 58 and 44 samples, respectively (Figure 1B). Results suggested that SIRT1 had no significant correlation to clinicopathological features such as age, gender, location and T-category. On the other hand, the altered SIRT1 expression was significantly correlated with the number of cancer sites (P=0.03), metastases (P=0.02) and metastatic sites (P=0.02). By using the Kaplan-Meier analysis, we found that strong SIRT1 expression of tumor tissue had an obvious association with poor prognosis in CRC patients (Log-rank test, P<0.01, Table 1, Figure 1C and D). Applying the multivariate Cox proportional hazards regression, SIRT1-Strong expression was significantly related to a lower survival rate, which displayed an independent relative risk of 2.459 compared with the weak group (P=0.003, Table 2). While gender, age and metastatic had no significant association with the CRC patient outcome. TNM-classification of T4 was the significant variable in the multivariate analysis with a relative risk of 2.762 compared with the T2 and T3 group (P=0.002). These data indicated that SIRT1 represented as an independent prognostic factor, which related to the prognosis of CRC patient.

Meanwhile, we detected the expression of a common stem cell marker CD133 (Human prominin-1) in these specimens. CD133 was expressed by various stem and progenitor cells originating from diverse sources, which was used as a stem cell marker universally. Double-labeling immunofluorescence analysis showed that CRC specimens with strong SIRT1 expression had more CD133-positive cells compared to those with weak SIRT1 expression (Figure 1E). This phenomenon demonstrated that SIRT1 had underlying association with CSCs in CRC.

**SIRT1 is overexpressed in CSC-like cells in CRC.** Then we attempted to confirm the association between SIRT1 and CSCs in CRC cell lines. Spheroid culture is a common method to enrich CSC-like cells. After the stem cell conditioned culture, mammospheres were harvested. Immunofluorescence staining analysis showed that the SIRT1 was enriched in mammospheres in SW620 and SW480 cells compared to the controls (Figure 2A). Meanwhile, the mRNA and protein levels of SIRT1 were obviously increased in the mammospheres compared to the adherent cells in SW620 and SW480 cells (Figure 2B and C).

We further evaluated the relationship between the expression of SIRT1 and CD133 in CRC. SW620 and SW480 were derived from

| Table 1 | Comparison of clinical characteristics between patients with weak and strong SIRT1 expression |
|---|---|---|---|---|
| Clinical factor | Weak | Strong | Total | P value |
| Age, years | | | | |
| Maximum | 85 | 83 | 168 | 0.46A |
| Minimum | 34 | 37 | 71 | 0.84B |
| Mean(SD) | 63(11.3) | 60(11.5) | 123 | 0.02C |
| Gender | | | | |
| Female | 25 | 20 | 45 | 0.49B |
| Male | 33 | 24 | 57 | 0.84B |
| Location | | | | |
| Colon | 4 | 5 | 9 | 0.49B |
| Rectum | 54 | 39 | 93 | 0.03B |
| No. of sites | | | | |
| 1 | 36 | 17 | 53 | 0.03B |
| ≥2 | 22 | 27 | 49 | 0.002C |
| T-category | | | | |
| I | 21 | 10 | 31 | 0.003 |
| II | 20 | 13 | 33 | 0.14C |
| III | 17 | 21 | 38 | 0.14C |
| IV | 15 | 22 | 37 | |
| Metastasis | | | | |
| Synchronous | 15 | 22 | 37 | |
| Metachronous | 18 | 13 | 31 | |
| Unknown or none | 25 | 9 | 34 | |
| Metastatic sites | | | | |
| Liver | 21 | 26 | 47 | 0.02 |
| Lung | 12 | 10 | 22 | 0.02 |
| Other or none | 25 | 8 | 33 | 0.02C |

A: T test; B: Fisher’s exact test; C: Chi-square test; +: The number of cancer glands at the same time of primary CRC surgery.
Figure 1 | SIRT1 expression in CRC tissue correlates with CRC patient survival and stem cell marker CD133 expression. (A) Immunohistochemical results. Images showed that the levels of SIRT1 expression in colorectal pericarcinomatous and cancer tissues. (B) Levels of SIRT1 staining were classified as weak or strong according to the percent of SIRT1+ glands. (C) Time to progression curve of CRC patients. Patients with strong SIRT1 expression showed a higher proportion of progression compared with the weak SIRT1 expression (P<0.01). (D) The overall survival curve of CRC patients. Patients with strong SIRT1 expression exhibited a reduced lifespan after tumor resection compared with the weak SIRT1 expression (P<0.01). (E) Double-labeling immunofluorescence analysis demonstrated that SIRT1 expression had a correlation with CD133 expression. Scale bars were shown. Results are representatives of three independent experiments.
CD133 three kinds of CRC cells (HCT116, SW620 and SW480) into the mRNA and protein levels (Figure 2E and F). We further separated SIRT1 expression of SW620 cells was higher than SW480 cells at both detected SIRT1 expression in these two cell lines. Results showed that no significant differences between CD133 regulated when cells were treated by NAM. Moreover, there were formation. Meanwhile, these abilities were apparently down-regulated when cells were treated by NAM in the abilities of colony and sphere formation in CRC cells.

SIRT1 deficiency reduces percentage of CD133+ cells and the formation of colony and sphere in CRC cells. According to the positive relationship between SIRT1 and CD133, we attempted to explore whether SIRT1 deficiency had an influence on the stemness of colorectal CSCs. Firstly, we used SIRT1 inhibitor nicotinamide (NAM, 20 mM) to pre-treat HCT116, SW620 and SW480 cells for 24 hours. Flow cytometry assay demonstrated that NAM significantly decreased the percentage of CD133+ cells in these three CRC cells (Figure 3A). Meanwhile, 20 mM NAM did not lead to the increase of apoptosis in these cells (figures were not shown). Then control cells and NAM pre-treated cells were seeded at 6-well plates in a density of 2000 cells/well. NAM pre-treated cells displayed lower ability of colony formation compared to the controls (Figure 3B). Then, we separated HCT116, SW620 and SW480 cells into the CD133+ cells and CD133- cells by magnetic bead sorting. These cells were detected their abilities of colony and sphere formation in the absence or presence of 20 mM NAM. The results showed that CD133+ cells had stronger abilities of colony and sphere formation. Meanwhile, these abilities were apparently down-regulated when cells were treated by NAM. Moreover, there were no significant differences between CD133+ cells treated with NAM and CD133- cells treated with NAM in the abilities of colony and sphere formation (Figure 3C and D).

To further confirm above findings, we used ShRNA to suppress the expression of SIRT1. HCT116 and SW620 cells were transduced with anti-SIRT1 lentivirus vectors (ShSIRT1-1, ShSIRT1-2) or scrambled shRNA (Scr-ShRNA). The shRNAs effective inhibition of SIRT1 expression was confirmed by western blotting analysis (Figure 4A). The flow cytometry results illustrated that the cells transduced with SIRT1 shRNAs remarkably decreased percentages of CD133+ cells in both HCT116 and SW620 cells (Figure 4B). The colony formation assay outcome indicated that the ability of colony formation distinctly dropped following with the down-regulation of SIRT1 (Figure 4C). Simultaneously, sphere formation ability also had a positive-correlation with the SIRT1 expression. SIRT1 knock-down obviously reduced the sphere formation in HCT116 and SW620 cells (Figure 4D). These data illustrated that SIRT1 inhibition significantly reduced the percentage of CD133+ cells and the abilities of colony and sphere formation in CRC cells.

SIRT1 deficiency results in lower CRC tumorigenicity in vivo. To further explore the impact of SIRT1 knockdown on tumor forming in vivo, SW620 cells were transduced with SIRT1 ShRNAs or Scr-ShRNA. These cells were injected subcutaneously into the nude mice. Results demonstrated that 2.0 × 106 cells infected with ShSIRT1-1 or ShSIRT1-2 were able to initiate the tumor formation, while 2.5 × 106 control SW620 cells were enough to initiate the tumor growth (Supplementary Table 1). Then 5.0 × 106 infected SW620 cells were injected subcutaneously into nude mice. The 5.0 × 106 SW620 cells were operated as control. The period of observation was from the 1st day after injection to the 18th day. The growth of tumors was monitored with measurements of two diameters in both mouse and human36. Meanwhile, SIRT1 down regulated p53 expression had an increase in SW620 when SIRT1 was knocked down in CRC cells. The result showed that SIRT1 knock-down cells exhibited obvious decrease of tumor volume and tumor weight compared with those of controls (Figure 5A). Then tumors were harvested on the 18th day (Figure 5B). Tumor further examination displayed that SIRT1 knock-down cells exhibited obvious decrease of tumor volume and tumor weight compared with those of controls (Figure 5C and D). These results demonstrated that SIRT1 knockdown using SIRT1 ShRNA decreased the tumorigenicity of CRC in vivo.

SIRT1 deficiency affects the expressions of p53 and stemness-associated genes in CRC. It was reported that p53 was involved in the formation of induced pluripotent stem (iPS) cell. The suppression of p53 increased the efficiency of iPS cell generation in both mouse and human36. Meanwhile, SIRT1 down regulated p53 activation in CML37. Therefore, we detected the expression of p53 when SIRT1 was knocked down in CRC cells. The result showed that p53 expression had an increase in SW620 when SIRT1 was knocked down (Figure 6A, B and C). Then we further explore the mechanism of SIRT1 in keeping the stemness by detecting the expression of several associated genes, including Oct4, Nanog, Sox2, Bmi-1, Cripto, Tert, Smo and Lin28. Quantitative real time PCR results showed that the mRNA levels of Oct4, Nanog, Cripto, Tert and Lin28 all showed apparent reductions when SIRT1 was inhibited.

Table 2 | Multivariate survival analysis

| Variable | Relative risk (95% confidence interval) | P value |
|----------|----------------------------------------|---------|
| SIRT1 Strong | 1 | | |
| Weak | 2.459 (1.315–4.469) | 0.003 |
| Gender Male | 1 | 1.321 (0.736–2.370) | 0.35 |
| Female | 1 | 0.548 (0.288–1.043) | 0.07 |
| Age ≥61 | 1 | 1.781 (0.915–3.466) | 0.09 |
| ≤60 | | | |
| Metastasis Yes | 1 | 2.762 (1.435–5.317) | 0.002 |
| No | | | |
| TNM stage T4 | 1 | | |
| T2+T3 | | | |

A: Presence of metastasis during the evaluating stage. B: Presence of none metastasis.
Figure 2 | Increased SIRT1 expression in colorectal CSC-like cells. (A) Immunofluorescence staining of SIRT1 in normal adherent cells and mammospheres of SW620 and SW480 cells. Scale bars (50 μm) were shown. (B) Quantitative real time PCR analysis of SIRT1 mRNA levels in normal adherent cells and mammospheres of SW620 and SW480 cells. (C) Western blotting and quantitative analysis of SIRT1 and GAPDH in normal adherent cells and mammospheres of SW620 and SW480 cells. (D) Representative flow cytometry plots and quantitative analysis showing the percentage of CD133<sup>+</sup> cells in SW620 and SW480 cells. (E) Quantitative real time PCR analysis of SIRT1 mRNA levels in SW620 and SW480 cells. (F) Western blotting and quantitative analysis of SIRT1 and GAPDH in SW620 and SW480 cells. (G) Western blotting and quantitative analysis of SIRT1 and GAPDH in CD133<sup>−</sup> and CD133<sup>+</sup> cells separated by CD133 microbeads or control cells of HCT116, SW620 and SW480 cells. All data are representative of three independent experiments. Significance: *P<0.05, **P<0.01, compared with the controls.
Figure 3 | Pharmacological inhibition of SIRT1 reduces percentage of CD133^+ cells and the colony formation in CRC cells. (A) Representative flow cytometry plots showing percentage of CD133^+ cells in control cells and cells treated with NAM (20 mM) of HCT116, SW620 and SW480 cells. (B) The colony formation analysis of control cells and cells treated with NAM (20 mM) of HCT116, SW620 and SW480 cells. (C) The colony formation analysis of CD133^+ cells and CD133^- cells in the absence or presence of 20 mM NAM in HCT116, SW620 and SW480 cells. (D) The sphere formation analysis of CD133^+ cells and CD133^- cells in the absence or presence of 20 mM NAM in HCT116, SW620 and SW480 cells. All data are representative of three independent experiments. Significance: *P<0.05, **P<0.01, ***P<0.001, compared with the controls.
Figure 4 | SIRT1 knockdown reduces percentage of CD133+ cells and represses colony and sphere formations in CRC cells. (A) Western blotting of SIRT1 and GAPDH in HCT116 and SW620 cells transduced with SIRT1 shRNAs (ShSIRT1-1 and ShSIRT1-2) or Scr-ShRNA. (B) Representative flow cytometry plots showing percentage of CD133+ cells in control cells and cells transduced with shRNAs or Scr-ShRNA of HCT116 and SW620 cells. (C) The colony formation analysis of control cells and cells transduced with shRNAs or Scr-ShRNA of HCT116 and SW620 cells. (D) The sphere formation analysis of control cells and cells transduced with shRNAs or Scr-ShRNA of HCT116 and SW620 cells. All data are representative of three independent experiments. Significance: **P<0.01, ***P<0.001, compared with the controls.
However, SIRT1 inhibition had no significant impact on the expressions of Sox2, Smo and Bmi-1 (Figure 6D). These results revealed that SIRT1 inhibition led to the increase of p53 expression and the decrease of several stemness-associated genes expressions in CRC cells.

**Discussion**

It has been well accepted that SIRT1 played a considerable role in many important biological processes such as apoptosis, senescence and metabolism. Here we reported that SIRT1 plays a crucial role in tumorigenesis of CRC by keeping the stemness of CSC-like cells. Immunochemical analysis demonstrated that SIRT1 was overexpressed in CRC tissues compared with their pericarcinomatous tissues. Moreover, CRC patients with high SIRT1 expression tended to exhibit poor prognosis. Meanwhile, we observed co-localization of SIRT1 with a stem marker CD133 in CRC tissues, indicating that SIRT1 had an underlying association with CSC-like cells of CRC. In vitro study showed that CSC-like cells had higher SIRT1 expression in CRC cell lines. More importantly, the stemness of CSC-like cells of CRC attenuated when SIRT1 was inhibited. Further studies revealed that inhibition of SIRT1 in CRC cells resulted in the increase of p53 expression and the decrease of several stemness-associated genes, including Oct4, Nanog, Cripto, Tert and Lin28.

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Figure 5 | SIRT1 knockdown using SIRT1 shRNA results in lower tumorigenicity in vivo. (A) The BALB/c nude mice were divided into four groups randomly, with control group (n=8) receiving 5 x 10⁶ SW620 cells and other groups receiving 5 x 10⁶ treated cells. Treated cells were SW620 cells which were transduced with SIRT1 shRNAs (ShSIRT1-1 and ShSIRT1-2) or Scr-ShRNA. The tumor growth rate of the experimental mice was measured by tumor volumes. Significance: **P<0.01, *P<0.05, means ShSIRT1-1 SW620 group vs. control SW620 group; ##P<0.01, #P<0.05, means ShSIRT1-2 SW620 group vs. control SW620 group. (B) Photographic illustration of tumor nodules excised from experimental mice on the harvesting time (18 days). (C) Tumor volumes of the experimental mice. The ShSIRT1-1 and ShSIRT1-2 groups were significantly decreased (*, P<0.05) compared to the controls. (D) Tumor weights of the experimental mice. The ShSIRT1-1 and ShSIRT1-2 groups were significantly decreased (*, P<0.05; ***, P<0.01) compared to the control.
The role of SIRT1 in cancer is under debate. Many previous studies have reported that high SIRT1 expression in some cancers, including prostate cancer, colorectal cancer, breast cancer, leukemia lymphoblasts, glioblastoma and skin carcinogenesis. In breast cancer, SIRT1 was reported to contribute to the tumorigenesis by inactivating tumor suppressor genes, such as cyclin G2. Inhibition of SIRT1 also caused gene re-expression of tumor suppressor genes with full retention of DNA hypermethylation. At the same time, the balance between SIRT1 and DBC1 was lost in various cancers, such as breast cancer and acute myeloid leukemia. On the other hand, RH Wang, et al. reported that activation of SIRT1 reduced tumorigenesis of various cancers via enhancing DNA damage response and maintain genome stability in mice model. Meanwhile, SIRT1 was lower expressed in HCC tissues compared to the corresponding pericarcinomatous tissues in the clinical samples. These researches indicate that SIRT1 may play different roles in the different kinds of cancers. Menssen A, et al. reported that in colorectal cancer, SIRT1 was activated by oncoprotein c-MYC and could promote c-MYC function in turn. The positive feedback loop formed by c-MYC, SIRT1 and DBC1 contributed to the tumor development. In our study, we also found that SIRT1 was overexpressed in colorectal tumor tissues compared to their pericarcinomatous tissues. More importantly, clinical data analysis showed that high SIRT1 expression of tumor tissue had a significant correlation with poor prognosis in CRC patients. Cox’s proportional hazards regression model further indicated that SIRT1 is an independent factor of prognosis of CRC patients. And the influence of SIRT1 on CSCs plays a crucial role on this phenomenon.

A lot of work has been done about the maintenance of characteristics of CSCs. Marhold M, et al. reported that HIF-1 alpha deregulated the PI3K/AKT/mTOR pathway of CSC to attenuate stem cells metabolism and growth in prostate cancer. Meanwhile, both Oct4 and Nanog overexpression enhanced the expression of EMT-related genes in CSC in breast cancer. The overexpression of Oct4 and Nanog accelerated the invasiveness of CSC. Our above results indicated that SIRT1 played an important role in keeping the properties of CSC-like cells in CRC. Further researches showed that the inhibition of SIRT1 in CRC cells resulted in the increase of p53 expression and reduction of various stemness-associated genes, including Oct4, Nanog, Cripto, Tert and Lin28.

p53, a critical tumor suppressor, plays an important role in tumorigenesis by regulating the expressions of several apoptosis-, cell cycle-, senescence-associated genes. Recent studies demonstrated that p53 had a strong impact on stem cells. p53 was considered as a crucial barrier of iPSC cell generation. Akita H, et al. also found that c-MYC enhanced the self-renewal capacity of liver CSCs in a p53-dependent manner.

Figure 6 | SIRT1 deficiency affects the expression of p53 and genes involved in self-renewal of SW620 cells. (A) Quantitative real time PCR analysis of p53 mRNA levels in control SW620 cells and cells transduced with shRNAs or Scr-ShRNA. (B) Western blotting of p53 and GAPDH of control SW620 cells and cells transduced with shRNAs or Scr-ShRNA. (C) Quantitative western blotting analysis of p53 protein levels in control SW620 cells and cells transduced with shRNAs or Scr-ShRNA. (D) Quantitative real time PCR analysis of the mRNA levels of stemness-associated genes (Oct4, Nanog, Cripto, Tert, Lin28, Sox2, Smo and Bmi-1) in control SW620 cells and cells transduced with shRNAs or Scr-ShRNA. All data are representative of three independent experiments. Significance: *P<0.05, **P<0.01, compared with the controls.
SIRT1 could inhibit p53 activation induced by genotoxic stress\(^\text{16}\). Wy Chen, et al. also reported that tumor suppressor HIC1 regulated p53-dependent DNA-damage responses by the modulation of SIRT1\(^\text{16}\). Previous study suggested that SIRT1 could regulate p53 activation by various pathways. As a class III histone deacetylase, SIRT1 can deacetylate some lysine residues of the tumor p53 protein, which leads to the instability and inactivation of p53\(^\text{17,18}\). Han, M. K. et al also reported that SIRT1 could upregulate Nanog expression in mouse ESCs by controlling ROS-related p53 subcellular localization\(^\text{19}\). Our research found that silencing SIRT1 led to the increases of mRNA and protein levels of p53 and the decrease of Nanog mRNA level in CRC cells. However, the clear mechanism remains to be confirmed.

SIRT1 has a complicated association with Oct4, which is an important transcription factor is frequently used as a marker for undifferentiated cells\(^\text{20}\). Low expression of Oct4 caused the differentiation of cells\(^\text{21}\). It has been reported that Oct4 can directly bind to the promoter region of SIRT1 to activate the SIRT1 expression\(^\text{22}\). Moreover, Oct4 could form Oct4-SIRT1-p53 axis to regulate pluripotency and DNA damage pathways to maintain the pluripotency and genomic stability of hESCs\(^\text{23}\). Our data showed that the inhibition of SIRT1 had a down-regulation on the expression of Oct4, which indicated there was a reciprocal regulation between SIRT1 and Oct4 through a feedback loop. By examining the published protein sequence analysis data (InterPro) of the function domain of SIRT1 protein, we found that SIRT1 has not DNA binding domain. It revealed that SIRT1 did not regulate the Oct4 expression by directly binding to Oct4 promoter. Additionally, Lingxia Wang, et al. reported that PCAF/SIRT1 balance played an important role in the regulation of Lin28 activity. Lin28 was acetylated by PCAF, which can be reversed by SIRT1. SIRT1 inhibitor NAM led to apparent decrease of Lin28 protein level\(^\text{24}\). Our result also demonstrated that Lin28 mRNA levels were reduced obviously when SIRT1 was knocked down in CRC cells. However, the underlying mechanisms by which SIRT1 regulates the mRNA expressions of these stemness-associated genes need to be further explored.

In conclusion, clinical samples analysis revealed that high expression of SIRT1 was associated with poor prognosis in CRC patients. Further study suggested that SIRT1 was overexpressed in CSC-like cells of CRC, and played a critical role in the tumorigenesis of CRC by maintaining stemness of CSC-like cells. All the results indicate that SIRT1 is a potential independent prognostic factor of CRC patients after tumor resection with curative intent, and reveals a promising treatment targeting CSCs in CRC.

**Methods**

**Ethics statement.** The procedure of human sample acquisition and experiments were approved by the Hospital Research Ethics Committees of Ren Ji Hospital. All animal studies were approved by the Animal Care Committee of Shanghai Jiao Tong University. And all methods were carried out in accordance with the approved guidelines.

**Clinical samples.** Colorectal cancer samples were obtained from patients in Department of Gastrointestinal Surgery, Ren Ji Hospital. From January 2006 to December 2013, patients who were confirmed as CRC were recruited for prospective follow-up. All subjects signed an informed consent form. All experimental protocols were conducted in accordance with the Declaration of Helsinki of the World Medical Association.

**Cell lines and culture.** Human colorectal carcinoma cell lines HCT116, SW620 and SW480 were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), where they were authenticated. SW480 were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), where they were authenticated.

**Cell transfection and selection.** HCT116 and SW620 cells were transduced with lentivirus vectors expressing SIRT1 shRNA. These cells were cultured for 24 hours, followed by the exposures to virus-containing supernatants (MOI = 20) via polybrene. Cells were selected by puromycin (2 μg/ml) (Sigma) 48 hours later. SHRNA1-1 and SHRNA1-2 were the anti-SIRT1 shRNA sequences. To turn off the effect of shRNA, a scramble sequence was used as a control. shRNA sequences were as follows:

| shRNA1-1: GAAGAGCTTCACGAATATTTA | shRNA1-2: GGTGAGCTTCATCTGTGTTA |
|-----------------------------------|----------------------------------|
| shScramble: GGGCGGCTTTTGGAGATTTG | shScramble: GGGCGGCTTTTGGAGATTTG |

**Real-time PCR analysis.** Total RNA was isolated from CRC cells using Trizol reagent method. 1 μg RNA was reversely transcribed into cDNA. The mRNA level was measured by real-time PCR. Primers used in this work were as follows:

| mRNA | sense primer | antisense primer |
|------|--------------|-----------------|
| TERT  | 5'-TGCTGGCCTAATAGAGTGGCA-3' | 5'-TGCTGGCCTAATAGAGTGGCA-3' |
| Bmi-1 | 5'-CGGGGTGACCTGTGAGCAT-3' | 5'-CGGGGTGACCTGTGAGCAT-3' |
| Nanog | 5'-GCGTTCTGTTGGCCTGAC-3' | 5'-GCGTTCTGTTGGCCTGAC-3' |
| Oct4  | 5'-TGTGCACCAACATCTACAGA-3' | 5'-TGTGCACCAACATCTACAGA-3' |
| Lin28 | 5'-TTCTCAGGAGAGAGCTTGC-3' | 5'-TTCTCAGGAGAGAGCTTGC-3' |
| Nanog | 5'-AATGGGCGGCTTGGAGACAT-3' | 5'-AATGGGCGGCTTGGAGACAT-3' |
| Bmi-1 | 5'-GGAAGCAGAAGATTTGTTCTTTG-3' | 5'-GGAAGCAGAAGATTTGTTCTTTG-3' |
| Lin28 | 5'-CATTGCCTGCTGGCCTGTTAAG-3' | 5'-CATTGCCTGCTGGCCTGTTAAG-3' |
| p53  | 5'-ATGCTGCTGTGACAGTCTACAG-3' | 5'-ATGCTGCTGTGACAGTCTACAG-3' |
| p53  | 5'-CCAGCATTTACACAGGGAAC-3' | 5'-CCAGCATTTACACAGGGAAC-3' |
| Tert  | 5'-GCGTTCTGTTGGCCTGAC-3' | 5'-GCGTTCTGTTGGCCTGAC-3' |
| Cripto | 5'-GGAGGCCAAGAAAGGGAATA-3' | 5'-GGAGGCCAAGAAAGGGAATA-3' |
| Tert reverse primer: GCGTTCTGTTGGCCTGAC-3' | 5'-TGTGCACCAACATCTACAGA-3' |
| Nanog reverse primer: GGTGAGCTTCACGAATATTTA | 5'-TGTGCACCAACATCTACAGA-3' |
| Nanog reverse primer: GGTGAGCTTCACGAATATTTA | 5'-TGTGCACCAACATCTACAGA-3' |
| Tert reverse primer: GGTGAGCTTCACGAATATTTA | 5'-TGTGCACCAACATCTACAGA-3' |
| Oct4 forward primer: GAGCAAAACCCGGAGGAGT-3' | 5'-TGTGCACCAACATCTACAGA-3' |
| Tert reverse primer: GGTGAGCTTCACGAATATTTA | 5'-TGTGCACCAACATCTACAGA-3' |
| Oct4 forward primer: GAGCAAAACCCGGAGGAGT-3' | 5'-TGTGCACCAACATCTACAGA-3' |
| Tert reverse primer: GGTGAGCTTCACGAATATTTA | 5'-TGTGCACCAACATCTACAGA-3' |
| Oct4 forward primer: GAGCAAAACCCGGAGGAGT-3' | 5'-TGTGCACCAACATCTACAGA-3' |
| Cripto reverse primer: CCGCCCCATAAATTCAAGAT-3' | 5'-TGTGCACCAACATCTACAGA-3' |
| Oct4 forward primer: GAGCAAAACCCGGAGGAGT-3' | 5'-TGTGCACCAACATCTACAGA-3' |
| Cripto reverse primer: CCGCCCCATAAATTCAAGAT-3' | 5'-TGTGCACCAACATCTACAGA-3' |

**Western blotting assay.** Cells were lysed in the cell lysates (Thermo) supplemented with protease inhibitors PMSF and Cocktail (Roche). Proteins were separated in 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose NC membranes (0.22 μm, Whatman). Membranes were blocked with blocking buffer (Li-COR), sequentially incubated in primary antibodies and secondary antibody. The primary antibodies included rabbit anti-human SIRT1 (Abcam) and rabbit anti-human GAPDH ( CST). The secondary antibody was Goat Anti-Rabbit IgG (Invitrogen). Protein levels were measured by gray value with Quantity One software.

**Immunofluorescence staining.** Cells were grown on sterile glass coverslips. After 24 hours, these cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% goat serum and sequentially incubated in primary antibody against SIRT1 (Rabbit monoclonal to SIRT1, Abcam) and Secondary Antibody FTC-conjugated Goat Anti-Rabbit IgG (CST). Cells were washed in PBS twice and stained with DAPI (Sigma). Finally, examined under a TCS_SP5 (Leica) confocal microscope.

**Coloncy formation assay.** The cells which more than 95% with viability were seeded in RPMI1640 with 10% FBS at a density of 2000 cells/well on 6-well plates (Corning). After 14 days, the plates were stained with crystal violet (Sigma) and counted the number of the colonies. The number of colonies was used to assess the ability of holoclone formation.

**Spheroid formation assay.** The cells were seeded at a density of 5000 cells/well on ultra low attachment 6-well plates (Corning). Cultures were cultured in serum-free DMEM/F12 supplemented with 100 μU/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml basic FGF, 20 ng/ml EGF, 2% B27 (Invitrogen). Cells with a three dimensional spherical structure (spheres) were collected 7 to 10 days later by gentle centrifugation (300 g/min, 3 min). The spheres were dissociated into single cells with trypsin-EDTA. The resulting single cells were re-suspended in the above medium to re-form spheres.

**Magnetic separation.** CD133 positive cells were isolated by magnetic bead sorting using the MidiMACS system (Miltenyi). The cells were prepared in a single-cell suspension in 300 μl of running buffer per 10⁷ total cells. Then cells were incubated with CD133 MicroBeads for 30 minutes in the refrigerator (2–8 °C). The CD133+ cells were enriched by LS MACS Column and MACS Separator as the manufacturer’s instructions. The purity of the cells was detected by flow cytometry (BD) using antibody against human CD133/255 (Miltenyi).
Tumorigenicity experiments in vivo. The SW620 cells and SW620 cells transduced with SIRT1 shRNAs or Scr-ShRNA were used in tumorigenicity experiments. The cells were prepared in suspensions of single-cell type using 100 μl PBS. 5.0 × 10^6 cells were injected into male BALB/c nude mice subcutaneously. Mice were examined once every 6 days. Tumor growth was evaluated by measuring the two diameters of the tumor nodules. Tumor volumes were calculated by the formula: Volume = π/6 × width × length × height (mm^3).

Immunohistochemistry analysis. The clinical samples were deparaffinized with xylene and rehydrated through graded alcohol series. For SIRT1 immunohistochemistry, antigen retrieval was conducted. Tissue sections were treated with microwave in 15 min in Citra Solution. 3% H2O2 (20 min) were used to block endogenous peroxidase. Tissue sections were sequentially blocked and incubated in 5% goat serum and primary antibodies. Primary antibodies against SIRT1 (Rabbit monoclonal to SIRT1, Sigma) (dilution 1:300) were applied and maintained overnight at 4 °C. Secondary antibodies anti-mouse IgG and anti-rabbit IgG (Invitrogen) were applied for 30 min at 37°C. The slides were visualized by DAB (5 min) and hematoxylin counterstain (Invitrogen).

Evaluation of SIRT1 immunohistochemistry. SIRT1 expression was evaluated on entire tissue sections of colorectal cancer specimens. Five fields of every section were viewed. Every specimen was given a score of 0, <50%, or ≥50% SIRT1+ expression. Strong was defined as a score of ≥50% SIRT1+ expression. Scores of 0 and <50% were considered to be weak. Immunohistochemistry results were evaluated independently by two skilled pathologists blinded from other data.

Statistics. All values from independent experiments were reported as the mean ±SEM. Student’s t-test analysis and One-way ANOVA were performed to determine statistical significance using GraphPad Prism software (Version 6.0). Log-rank test was performed for categorical data of survival rates analysis using GraphPad Prism. Multivariate analysis was conducted on the Cox regression model. Statistical procedures were performed using SPSS. Statistical significance was set at p < 0.05.

Acknowledgments

This project was supported by the Key Basic Research Project of China (Grant NO. 2011CB606603); Major State Scientific Research Program of China (Grant NO. 2007CB815702).
Author contributions

X.J.C. and K.S. contributed equally to this manuscript. X.J.C., K.S., M.Z. and L.X.W. conceived and designed the study. X.J.C., S.F.J. and N.C. performed animal experiments. X.J.C. and X.Z. performed cell experiments. H.B.Z. and K.S. performed flow cytometry experiments. Z.S.W. collected the colorectal cancer samples. Y.X.X. and K.S. performed the statistical analysis of the clinical samples. X.J.C. wrote the draft manuscript. Z.M. and L.X.W. finalized the manuscript. All authors read and approve the final manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Chen, X. et al. High levels of SIRT1 expression enhance tumorigenesis and associate with a poor prognosis of colorectal carcinoma patients. Sci. Rep. 4, 7481; DOI:10.1038/srep07481 (2014).

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