Knockout of MARCH2 inhibits the growth of HCT116 colon cancer cells by inducing endoplasmic reticulum stress

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Membrane-associated RING-CH protein 2 (MARCH2), a member of the MARCH family, functions in vesicle trafficking and autophagy regulation. In this study, we established MARCH2 knockout HCT116 cell lines using CRISPR/Cas9-mediated genome editing to evaluate the role of MARCH2 in colon cancer in vitro and in vivo. Knockout of MARCH2 suppressed cell proliferation, and promoted autophagy, apoptosis and G2/M phase cell cycle arrest. These effects were associated with activation of endoplasmic reticulum (ER) stress. In addition, loss of MARCH2 sensitized HCT116 cells to the chemotherapy drugs etoposide and cisplatin. Moreover, we analyzed the clinical significance of MARCH2 in human colon carcinoma (n = 100). High MARCH2 expression was significantly associated with advanced clinicopathological features and poorer overall survival in colon carcinoma. MARCH2 expression correlated negatively with expression of the unfolded protein response molecule p-PERK in colon cancer. Collectively, these data reveal a relationship between MARCH2, ER stress and colon cancer, and indicates MARCH2 may have an important role in the development and progression of colon cancer.

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The endoplasmic reticulum (ER) is a cellular membrane compartment in eukaryotic cells involved in the synthesis and sorting of secretory and membrane proteins that is also an important site for initiation of autophagosome formation.1-3 Many different perturbations can alter the function of ER, leading to de accumulation of unfolded or misfolded proteins inside the ER, a cellular referred to as ER stress. ER stress initiates a series of adaptive mechanism that together are known as the unfolded protein response (UPR).3

The ER stress is a complex process that involves activation of three major signaling pathways: inositol-requiring enzyme-1α (IRE1α), activating transcription factor 6 (ATF6) and protein kinase RNA-like endoplasmic reticulum kinase (PERK). In response to the accumulation of unfolded proteins in the ER, GRP78/BiP is released from IRE1α, ATF6 and PERK to chaperone the accumulated proteins to degradation via ubiquitination.4 GRP78/BiP-free ER stress sensors are differentially activated to initiate their downstream cascades. Activated IRE1α acts as an RNase to process the mRNA encoding XBP1, leading to the expression of an active transcription factor (XBP1s, s correspond to splicing). XBP1s functions as a transcriptional activator for UPR gene targets such as GRP78/BiP and calreticulin.5,6 Concomitantly, during ER stress, ATF6α is released from GRP78/BiP and translocates from the ER to Golgi where it undergoes cleavage. Cleaved ATF6α translocates to the nucleus and transactivates various chaperones and major ER stress markers such as the CAAT-enhancer binding protein (CHOP) gene.6 Moreover, increased expression of CHOP has been reported to activate apoptosis in various studies.7

The PERK/EIF2α pathway is a component of the UPR signaling pathway: when no ER stress is present, PERK is combined with GRP78/BiP in an inactive state; under ER stress conditions, PERK separates from its molecular chaperone GRP78/BiP and becomes activated, and phosphorylates and inactivates EIF2α leading to termination of the majority of cellular protein synthesis, which in turn regulates the cell cycle. The PERK/EIF2α pathway also activates ATF4, which upregulates CHOP expression.8 CHOP is a specific transcription factor of ER stress, which induces the expression of the ER stress-related protein CK1 and genes related to cell cycle regulation.9

Membrane-associated RING-CH protein 2 (MARCH2), contains a RING domain that exerts E3 ubiquitin ligase activity.10 MARCH2 was first described as a member of the ubiquitin ligase family probably related to viral immune evasion proteins.11 MARCH2 participates in vesicle trafficking by interacting with syntaxin 6.12 As an E3 ubiquitin ligase, MARCH2 can ubiquitinate several substrates, such as DLG1,13 β2AR14 and CFTR.15 In our previous study, we demonstrated that MARCH2 negatively regulates cell autophagy.16 This mechanism involves inhibition of the PI3CA-AKT-MTOR and CFTR signaling pathways. However, the role of MARCH2 in tumor remains unclear.

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Clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) is a newly developed biotechnology for editing genomic DNA. The CRISPR/Cas9 system consists of two components: a single guide RNA (sgRNA) that provides target specificity and a CRISPR-associated protein (Cas9) that functions as a helicase and endonuclease. Cas9 unwinds and cleaves double-stranded DNA containing the protospacer adjacent motif (NGG; N indicates any base) through the sgRNA. The non-homologous end-joining DNA repair pathway is then activated to repair the lesion, generating errors that eventually interrupt the open reading frame and lead to dysfunctional gene expression at the transcriptional level. In this paper, we demonstrate that knockout of MARCH2 using CRISPR/Cas9 gene editing biotechnology suppressed the growth of colon cancer cells in vitro and in vivo via effects associated with the ER stress pathway.

Results

Knockout of MARCH2 using CRISPR/Cas9-mediated genome editing inhibits cell proliferation. To clarify the function of MARCH2 in colon cancer, we knocked out MARCH2 in HCT116 colon cancer cells. Through a series of screens, three Cas9-MARCH2 clones were selected. Sequence analysis revealed the three clones, clone 1, GT → GCT; clone 2, AGGTCG → AG; clone 3, TCGTG → C, contained in-frame shift mutations which disrupted the MARCH2 ORF, leading to deletion of the transmembrane, RING or PDZ functional domains (Supplementary Figure 1a–c). Western blotting indicated MARCH2 protein was not detectable in Cas9-MARCH2 HCT116 cells (Figure 1a).

Next, we tested the viability of the Cas9-MARCH2 HCT116 cells. Time MTS course assays confirmed clone 1, clone 2 and clone 3 Cas9-MARCH2 HCT116 cells had reduced cell viability compared with control cells (Figure 1b). EdU (5-ethyl-2'-deoxyuridine) is an alternative to the BrdU assay for directly measuring active DNA synthesis or S phase synthesis during the cell cycle. Clone 1, clone 2 and clone 3 Cas9-MARCH2 HCT116 cells contained lower percentages of EdU-positive cells (i.e., proliferative cells) than control cells (Figures 1c and d). Colony formation assays demonstrated knockout of MARCH2 suppressed the colony-forming ability of HCT116 cells (Figures 1e and f). Among the three clones, the most obvious inhibitory effects were observed for clone 3, so this clone was selected for all subsequent experiments.

Knockout of MARCH2 promotes apoptosis and cell cycle arrest in the G2/M phase. FITC–Annexin-V and PI staining revealed knockout of MARCH2 in HCT116 cells increased the number of apoptotic cells, with a time-dependent effect observed (Figures 2a and b). To determine whether knockout of MARCH2 enhanced apoptosis via the caspase-dependent pathway, Cas9-MARCH2 HCT116 cells were pretreated with the pan-caspase inhibitor z-VAD-fmk for 2 h, then further cultured for 24 or 48 h. Flow cytometry analysis revealed pretreatment with z-VAD-fmk partly reduced the percentage of apoptotic cells. Western blotting analysis confirmed knockout of MARCH2 increased the levels of cleaved caspase-3 and PARP, whereas z-VAD-fmk pretreatment attenuated their cleavage in Cas9-MARCH2 HCT116 cells (Figures 2c and d). These results demonstrate caspase-dependent signaling mediates the increased levels of apoptosis in Cas9-MARCH2 HCT116 cells.

Based on the preliminary assays in which we determined the effects of MARCH2 KO on cell viability and apoptosis, we explored the effect of MARCH2 inactivation on cell cycle progression by PI staining. As shown in Figures 3a and b, a significantly higher percentage of cells in G2/M-phase were observed in Cas9-MARCH2 HCT116 cells than in control groups both at 24 h and 48 h. There was a concomitant reduction in the proportion of cells in the S phase at 24 h and GO/G1 phase at 48 h. These data suggest that inhibition of cell growth in Cas9-MARCH2 HCT116 cells may be associated with the induction of G2/M-phase cell cycle arrest.

The G2/M transition is regulated by the cyclin B1/CDC2 complex and MDM2 is a negative regulator of p21 involved in the G2/M checkpoint that is required for cell cycle arrest in the G2/M phase. Further investigation indicated that knockout of MARCH2 decreased the expression of CDC2, Cyclin B1 and MDM2 (Figures 3c and d). This finding suggested that MARCH2 KO-induced G2/M phase arrest may be related to the decrease of cyclin B1/CDC2 activities.

ER stress is associated with autophagy, apoptosis and cell cycle arrest in Cas9-MARCH2 HCT116 cells. As MARCH2 localizes to the ER, we examined whether ER stress exists in Cas9-MARCH2 HCT116 cells. Western blotting revealed knockout of MARCH2 in HCT116 cells upregulated UPR molecules including phosphorylated PERK (Thr 981), EIF2α (Ser 51) and ATF4 (Figure 4a, lane 2 versus lane 1). This effect was accompanied by a significant increase in the amount of ATG12-ATG5 conjugation in Cas9-MARCH2 HCT116 cells. In contrast, overexpression of MARCH2 decreased the levels of p-PERK, p-EIF2α, ATF4, CHOP and ATG12-ATG5 in HCT116 cells (Figure 4a, lane 4 versus lane 3). In addition, we also observed the slight increase of cleaved ATF6, p-IRE1α (Ser 724) and XBPs1 in Cas9-MARCH2 HCT116 cells (Supplementary Figure 2). Consistent with these findings, levels of the ER stress sensor protein GRP78, Calnexin and calreticulin were upregulated in Cas9-MARCH2 HCT116 cells (Supplementary Figure 2). These results suggested that MARCH2 KO-mediated activation of PERK, ATF6 and IRE1α pathways differentially contributed to MARCH2-mediated autophagy. Prolonged activation of ER stress often leads to cellular apoptosis that is reflected by increased levels of the protein CHOP. Consistent with our expectation, the increased expression of CHOP was observed in Cas9-MARCH2 HCT116 cells (Figure 4a, lane 2 versus lane 1), thereby implicating appearance of cellular apoptosis in these cells.

To provide further evidence that MARCH2 KO upregulates ER stress, we investigated the effect of treating HCT116 cells with dithiothreitol (DTT) to induce cellular ER stress. DTT-induced ER stress was confirmed by increased expression of CHOP (Figure 4b, lane 1 and lane 5). In the presence of DTT, knockout of MARCH2 increased the levels of CHOP and decreased the levels of SQSTM1 (Figure 4b, lane 2 versus lane 1, lane 4 versus lane 3), indicating loss of MARCH2...
enhanced DTT-mediated ER stress and autophagy. Over-expression of MARCH2 in HCT116 cells downregulated CHOP and increased the accumulation of SQSTM1 (Figure 4b, lane 6 versus lane 5, lane 8 versus lane 7), suggesting MARCH2 activity impairs DTT-mediated ER stress and autophagy. These findings indicate MARCH2 regulates autophagy, at least in part in association with ER stress signaling.

To further confirm the association with ER stress, Cas9-MARCH2 HCT116 cells were treated with salubrinal, which acts as a phosphatase inhibitor and increases EIF2α phosphorylation leading to global decrease in translation, subsequently lessening ER stress.23 As shown in Figure 4c (lane 4 versus lane 2), salubrinal reduced the levels of CHOP, accompanied by decreased expression of LC3B-II, cleaved caspase-3 and cleaved-PARP. Moreover, the levels of CDC2, Cyclin B1 and MDM2 were enhanced in the presence of salubrinal (Figure 4c, lane 4 versus lane 2). The results implied that the ER stress response is upstream of autophagy, apoptosis and G2/M phase arrest in Cas9-MARCH2 HCT116 cells.

To further validate the role of ER stress in MARCH2 KO-mediated induction of autophagy, we employed a genetic approach targeting PPP1R3A (protein phosphatase 1, regulatory subunit 3A), which is responsible for EIF2α dephosphorylation.24 As shown in Figure 4d, transfection of PPP1R3A siRNA significantly decreased PPP1R3A protein expression at 48 h. Correspondingly, the levels of the downstream protein CHOP were reduced in Cas9-MARCH2 HCT116 cells transfected with PPP1R3A siRNA (Figure 4d, lane 4 versus lane 2), implying that PPP1R3A silencing inhibited ER stress signaling. Simultaneously, PPP1R3A deficiency attenuated the expression of LC3B-II, cleaved caspase-3 and cleaved-PARP, but enhanced the levels of CDC2, Cyclin B1 and MDM2, indicating that knockdown of PPP1R3A attenuated autophagy, apoptosis and G2/M phase arrest in Cas9-MARCH2 HCT116 cells (Figure 4d, lane 4 versus lane 2). In view of the fact that the levels of PPP1R3A were increased in Cas9-MARCH2 HCT116 cells (Figure 4d, lane 2 versus lane 1), thus implying that increased PPP1R3A may be one of the mechanisms in MARCH2 KO-induced ER stress.

Next, we analyzed the phenotypes of salubrinal-treated Cas9-MARCH2 HCT116 cells. Compared with control cells, knockout of MARCH2 increased GFP-LC3B puncta distribution in the presence of BafA1 alone, whereas salubrinal reversed this effect (Supplementary Figure 3a and b). Flow cytometry analysis revealed salubrinal reversed the increase in apoptosis observed in Cas9-MARCH2 HCT116 cells (Supplementary Figure 3c and d). Likewise, salubrinal blocked the G2/M phase arrest induced by knockout of MARCH2 (Supplementary Figure 3e). Taken together, these findings

Figure 1 Knockout of MARCH2 suppresses colon cancer cell growth. (a) Western blot analysis of MARCH2 protein expression in Cas9-MARCH2 HCT116 cells. (b) MTS cell viability assay. Control (wild-type) and Cas9-MARCH2 HCT116 cells were seeded in 96-well plates (3000 cells/well; five replicates), serum-starved for 18 h and then pulsed with 10% FCS for 24 h, 48 h, 96 h or 144 h. Data are mean ± S.D. of three independent experiments. (c) Representative confocal microscopy of immunofluorescent staining for EdU. Control and Cas9-MARCH2 HCT116 cells were plated on glass slides in 24-well plates, serum-starved for 18 h, pulsed with 10% FCS for 48 h and incubated with EdU for 4 h. Nuclei were stained with Hoechst 33342. Scale bar: 100 mm. (d) Quantification of the percentage of EdU-positive cells (in 200 cells). Each bar represents the mean ± S.D. of three independent experiments. (e) Representative images of colony formation by control (wild-type) cells and Cas9-MARCH2 HCT116 cells. (f) Quantitative analysis of colony numbers for three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.
underpin the importance of ER stress responses in the growth arrest induced by knockout of MARCH2 in HCT116 cells.

Knockout of MARCH2 sensitizes HCT116 cells to chemotherapy. Given that knockout of MARCH2 induced growth arrest, we investigated whether knockout of MARCH2 enhanced the chemosensitivity of colon cancer cells. Etoposide and cisplatin are clinically used for the treatment of several cancers, including colon cancer. Cas9-MARCH2 HCT116 cells were treated with different concentrations of cisplatin or etoposide for 24 h. MTS assays revealed etoposide and cisplatin reduced the cell viability of HCT116 cells in a dose-dependent manners, and these suppressive effects were enhanced in Cas9-MARCH2 HCT116 cells (Figures 5a and b). Flow cytometry showed that the number of apoptotic cells was higher in cisplatin (10 μM)- or etoposide (30 μM)-treated Cas9-MARCH2 HCT116 cells than control cells treated with etoposide or cisplatin alone (Figures 5c and d). Western blot analysis showed both etoposide and cisplatin elevated LC3B-II accumulation in control HCT116 cells (Figures 5e and f, lane 4 versus lane 2, lane 8 versus lane 6). These findings suggest MARCH2 inactivation-mediated autophagy enhanced the sensitivity of colon cancer cells to chemotherapy.

Overexpression of MARCH2 enhances the tumorigenicity of colon cancer cells in vivo. To investigate the influence of overexpressing MARCH2 on colon tumor cell proliferation in vivo, three colon cancer cell lines (HCT116, LOVO and HT-29) stably overexpressing MARCH2 were established. MTS assays indicated overexpression of MARCH2 enhanced cell viability (Supplementary Figure 4a and b) and increased the percentage of EdU-positive cells in HCT116 cells, LOVO cells and HT-29 cells (Supplementary Figure 4c and d). These data indicate overexpression of MARCH2 promotes the growth of colon tumor cells. Next we investigated the sensitivity of MARCH2-overexpressing cells to chemotherapeutic drugs. As shown in Supplementary Figure 5a and 5b, both etoposide and cisplatin reduced HCT116 cell viability in a dose-dependent manners, but this suppressive effect could be partially reversed in MARCH2-overexpressing HCT116 cells. The similar results occurred in the detection of apoptosis (Supplementary Figure 5c and 5d), indicating that
MARCH2 overexpression decreased the sensitivity of colon cancer cells to chemotherapy.

The *in vivo* effects of MARCH2 were evaluated using a colon cancer xenograft model established in BALB/C nude mice. Mice were subcutaneously injected in the right axilla with empty vector-transfected HCT116 cells, stable MARCH2-overexpressing HCT116 cells, MARCH2 wild-type HCT116 cells or Cas9-MARCH2 HCT116 cells. At 20 days after inoculation, the mice were sacrificed and the tumors in each mouse were excised and photographed. As shown in Figure 6a, the stable MARCH2-overexpressing group had larger tumors than the vector group, indicating MARCH2 enhanced the tumorigenicity of HCT116 cells. However, the Cas9-MARCH2 group had smaller tumors than the control group, indicating knockout of MARCH2 reduced the tumorigenicity of HCT116 cells (Figure 6a).

Next, we examined the expression of MARCH2, Ki-67, SQSTM1 and cleaved caspase-3 in the xenograft tumor sections. Immunohistochemistry revealed MARCH2-overexpressing tumors contained a significantly higher proportion of Ki-67-positive cells than vector control tumors (Figures 6b and c), whereas Cas9-MARCH2 tumors contained fewer Ki-67-positive cells (Figures 6b and c, lower panel). Simultaneously, SQSTM1 expression was stronger in MARCH2-overexpressing tumors and weaker in Cas9-MARCH2 tumors than control group. There was no significant difference in cleaved caspase-3 expression between the MARCH2-overexpressing tumors and vector control group; however, cleaved caspase-3 staining was stronger in Cas9-MARCH2 tumors than vector control tumors (Figures 6b and c, lower panel). Collectively, these data suggest MARCH2 knockout-mediated autophagy and apoptosis exerted an anti-tumor effect in HCT116 cells *in vivo*.

**Correlation between MARCH2 expression and the clinicopathologic characteristics of colon cancer.** We next examined the expression of MARCH2, SQSTM1, p-PERK, p-EIF2α and p-IRE1α in primary colon cancer tissues and adjacent non-tumor tissues. Weak MARCH2 signals, mainly localized in the cytoplasm, were detected in non-tumor tissues. In contrast, strong or moderate expression of MARCH2 was observed in the majority of colon cancer tissues (Figure 6a, Supplementary Figure 6). Similarly to MARCH2, the expression of SQSTM1 was higher in colon cancer tissues than non-tumor tissues. In contrast, strong or moderate expression of p-PERK (Thr 981) and p-EIF2α (Ser 51) were observed in the non-tumor tissues adjacent to cancer, with weak or absent signals in colon cancer tissues. There was no significant difference in the expression of p-IRE1α between colon cancer tissues and the non-tumor tissues adjacent to cancer (Supplementary Figure 7). These data further imply that high expression of MARCH2 is accompanied by decreased PERK/EIF2α signaling and autophagy.
We next analyzed the relationship between MARCH2 expression and the clinicopathologic characteristics of 100 patients with colon cancer (Table 1). High MARCH2 expression was significantly associated with larger tumor size (odds ratio (OR) = 12.27, \( P < 0.001 \)), histological grade (OR = 6.58, \( P = 0.004 \)), interstitial invasive depth (OR = 23.33, \( P < 0.001 \)), advanced tumor stage (OR = 5.22, \( P = 0.012 \)) and positive lymphatic metastasis (OR = 11.84, \( P < 0.001 \)). These data suggest high level MARCH2 expression is positively associated with malignant potential in colon cancer.

We also assessed the association between MARCH2 expression and survival; 100 patients with colon cancer were followed-up for up to 98 months. During the observation period, 60 (60%) patients died of recurrence. The 8-year cumulative survival rate for all patients was 40%. Patients with moderate to high expression of MARCH2 (++) to (++; Supplementary Figure 6) had poorer overall survival compared with patients with negative to low expression of MARCH2 (− to +; Figure 7b). In contrast, patients with negative to low expression of p-PERK had poorer overall survival compared with patients with moderate to high expression of p-PERK. Furthermore, correlation analysis demonstrated MARCH2 expression correlated negatively with the levels of p-PERK in colon carcinoma (Table 2 and Supplementary Figure 6).

Discussion

In this study, we investigated the function and mechanism of action of MARCH2 during the development of colon cancer in vitro and in vivo. Overexpression of MARCH2 promoted the proliferation of HCT116 cells, whereas knockout of MARCH2 suppressed cell viability and promoted apoptosis and G2/M phase cell cycle arrest. Further investigation revealed these effects were closely associated with ER stress in MARCH2 knockout cells, via a mechanism involving activation of PERK-EIF2α-ATF4-CHOP signaling. Importantly, knockout of MARCH2 increased the sensitivity of colon cancer cells to
etoposide or cisplatin. Moreover, high MARCH2 expression was associated with poorer overall survival in colon cancer, indicating that expression of MARCH2 may represent an independent prognostic factor for overall survival. In addition, overexpression of MARCH2 was accompanied by decreased autophagy, as indicated by reduced LC3B expression and enhanced SQSTM1 expression, and decreased ER stress signaling, as reflected by reduced PERK and ATF6 levels. This is the first evidence of a relationship between MARCH2, ER stress and autophagy in colon carcinoma, and indicates inactivation of MARCH2 may represent a novel therapeutic target for colon cancer.

The ER stress pathway has an important role in the development of cancer. High level activation of UPR molecules is observed in a wide range of primary human tumors, including glioblastoma and carcinomas of the breast, stomach, esophagus, and liver. In contrast, somatic mutations in IRE1α or PERK, in most cases loss-of-function mutations, are rarely (<1%) detected in these tumors. Despite the overwhelming evidence for ongoing ER stress and UPR activation in many forms of cancer, whether this ultimately inhibits or promotes tumor growth remains an area of intense debate. Most evidence arguing that the UPR supports tumor growth comes from xenograft studies in mice, in which genetic deletion of one or more branches of the UPR or altering the expression of ER chaperones reduces the growth of tumor cells in vivo. Xie et al. reported that mild ER stress through BiP-PERK-EIF2α pathway has a critical role in protecting against RIBE-induced cellular damage and hence may potentially decrease the risk of secondary cancer after radiotherapy. Pytel et al. showed PERK is a haploinsufficient tumor suppressor, as the gene dose determines the tumor-suppressive versus tumor-promoting properties of PERK in melanoma. Here, we proved inactivation of MARCH2 in HCT116 cells increased GRP78/BIP, subsequent PERK/EIF2α signaling and the levels of downstream CHOP. The immunohistochemistry results suggested that MARCH2 KO-mediated PERK/EIF2α pathway may contribute to tumor suppression. How MARCH2 KO affects ER stress remains unclear, which deserves further investigation in the future. As an E3 ligase localized in the ER, MARCH2 may maintain the ER homeostasis under normal circumstances via unknown mechanism. Under MARCH2 inactivation, such homeostasis maybe had broken, which caused the accumulation of unfolded or misfolded proteins in the lumen of the ER. Molecular chaperones such as GRP78/BIP dissociated from the UPR sensors, leading to their activation. We will further explore this hypothesis. Similarly, the relationship between MARCH2 and ER stress in colon carcinoma also needs further investigation.

Elevated SQSTM1 is considered a hallmark for impaired autophagy and has been associated with poor prognosis in some tumor types. Burdelski et al. found high levels of SQSTM1 were significantly associated with increased tumor cell proliferation in prostate cancer. New evidence supports a key autophagy-independent role for the adaptor SQSTM1 in the signaling functions central to tumor initiation in the epithelium and suppression of tumor progression in the stroma. Schlaffi et al. reported the autophagy markers LC3 and SQSTM1 had prognostic value in early-stage non-

Figure 5 Knockout of MARCH2 sensitizes HCT116 cells to chemotherapy. (a, b) Control cells and Cas9-MARCH2 cells were cultured in 96-well plates (5000 cells/well, five replicate wells), treated with indicated dose of cisplatin or etoposide for 24 h. Cell viability was assessed using the MTS assay. The IC50 of etoposide or cisplatin is 17.2 μM or 40.8 μM, respectively. Data are mean ± S.D. of three independent experiments. (c, d) Control cells and Cas9-MARCH2 HCT116 cells were treated with or without etoposide or cisplatin for 24 h. Apoptosis was quantified by Annexin-V/PI staining and flow cytometry. (e) Western blot of the levels of LC3B-II in cells treated as described in (c). (f) Histogram of the of LC3B-II/ACTB ratio for cells treated as described in e. The average value in the control group was normalized to 1. Data are mean ± S.D. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001
small cell lung cancer. In this study, high MARCH2 expression was strongly associated with the clinicopathological features of aggressive disease, including larger tumor size, higher histological grade, deeper interstitial invasion, advanced tumor stage and positive lymph node metastasis. Furthermore, high MARCH2 expression correlated with increased levels of SQSTM1, supporting the hypothesis that MARCH2-regulated autophagy has an important role in the development of colon cancer. Therefore, SQSTM1 and MARCH2 may represent prognostic biomarkers of malignant change in colon cancer. Moreover, as knockout of MARCH2 sensitized colon tumor cells to etoposide or cisplatin treatment, this approach merits further exploration as a therapeutic strategy for colon cancer.

Materials and Methods

Antibodies and reagents. The following antibodies were used: Rabbit anti-mouse p62/SQSTM1 antibody (MBL International, Woburn, MA, USA; PM045), anti-LC3B (Sigma Aldrich, St. Louis, MO, USA; L7543), anti-Ki-67 (Pierce, Appleton, WI, USA, PA516446), anti-ATG5 (Cell Signaling Technology, Danvers, MA, USA, 12994 S), anti-CHOP (Cell Signaling Technology, 2895 P), anti-GRP78 (Bioss, Scotland, UK, bs-1219 R), anti-PERK (Abcam, Cambridge, UK, ab65142), anti-Phospho-PERK (Santa Cruz Biotechnology, California, CA, USA, sc-32577), anti-EIF2α (Cell Signaling Technology, 5324 P), anti-Phospho-EIF2α (Cell Signaling Technology, 3398 S), anti-IRE1α (Abcam, AB124945), anti-XBP1s (Biolegend, California, CA, USA, 647501), anti-ATF6α (Santa Cruz Biotechnology, sc-54599), monoclonal anti-ATF6 antibody (Imagex, Littleton, CO, USA, IMG-273), anti-Calnexin (AF-18) (Abcam, ab31290), anti-Calreticulin (Abcam, ab4), anti-ATF4 (Cell Signaling Technology, 11815 S), anti-CDC2 (Santa Cruz Biotechnology, sc-54), anti-MDM2 (Abcam, ab137413), anti-cyclin B1 (Santa Cruz Biotechnology, sc-245), anti-cleaved-PARP (Cell Signaling Technology, 5625 S), anti-cleaved caspase-3
Abcam, ab52293), anti-PPP1R3A (Abcam, ab104986). Secondary antibodies included DyLight 800/DyLight 680-conjugated IgG against mouse (Rockland, Philadelphia, PA, USA, 610-145-002/610-144-002) or rabbit (Rockland, 611-145-002/611-144-002). Other reagents used in this study were: Bafilomycin A1 (BafA1, Sigma Aldrich, B1793), DTT (DTT, Merck, Kenilworth, NJ, USA, 233155), 3-MA (Sigma Aldrich, M9281), Z-VAD-FMK (Santa Cruz Biotechnology, sc-311561), Hoechst 33342 (Sigma Aldrich, 14533).

**Cell culture, transfection and treatments.** HCT116, LOVO, HT-29 cells were cultured in DMEM (12800-017; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified incubator with 5% CO₂. Cells were transfected with pcDB-MARCH2 or empty vector plasmids using MegaTran 1.0 Transfection Reagent (TT200004; ORIGEN, Rockville, MD, USA) according to the manufacturer’s instructions, and selected using G418 to establish MARCH2-overexpressing cell lines; empty vector-transfected cells were generated as a control.
Knockout of MARCH2 using CRISPR/Cas9-mediated genome editing. A MARCH2 knockout HCT116 cell line was established by CRISPR/Cas9-mediated genome editing. The target sequences for CRISPR interference were designed by Shanghai Bimodel Organization Science & Technology Development Co., Ltd., Shanghai, China. The target sequence for human MARCH2 was TCCAAGTCGGTGGAGGCTAGGGG (Exon 2). The clones were screened by sequencing.

Cell viability assay. Cell viability assays were performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (G3582; Promega, Madison, WI, USA) according to the manufacturer’s protocols. Cell viability was calculated as follows: cell viability = absorbance of test group/absorbance of control cell group ×100%. Each experiment was performed in three replicate wells and independently repeated three times.

EdU incorporation assay. Cell proliferation was detected using the EdU detection kit (C10639; Invitrogen) according to the manufacturer's protocol. Briefly, cells were plated on coverslips and EdU was added to the medium 4 h before harvesting cells. Cells were fixed in 4% paraformaldehyde and nuclei were counterstained with Hoechst 33342. Flow cytometric analysis. To assess the cell cycle, cells were seeded into six-well plates, incubated for different times, collected, fixed in 70% ice-cold ethanol overnight, incubated in RNase (100 μg/ml) for 30 min, stained with PI (50 μg/ml) and analyzed using a FACScalibur flow cytometer (Becton Dickinson, Franklinlakie, NJ, USA). During the analysis of cell cycle, the death cells and cell debris and aggregates were excluded. We only analyzed 'gated' cells.

Immunofluorescence, fluorescence and confocal microscopy. Cells were cultured in confocal dishes and treated as indicated, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (ST795; Beyotime, Shanghai, China), The dishes were then incubated with FBS overnight and exposed to primary antibody for 1 h at 4 °C. After washing three times with phosphate-buffered saline, the dishes were incubated with FITC-conjugated or Alexa Fluor 680 secondary antibody, and imaged using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Western blot. Total cellular proteins were extracted using radio immunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, with freshly-added proteinase inhibitor cocktail and phosphatase inhibitors). Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, then membranes were blocked and incubated with the indicated antibodies. Blots were visualized using an IRDye 800CW-conjugated or Alexa Fluor 680 secondary antibody, and imaged using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. SPSS 15.0 software (Chicago, IL, USA) was used for statistical analysis. The p-test was employed for comparisons between groups, and correlations between two variables were evaluated using the Spearman’s rank correlation test. P < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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