TRIM25 Regulates Oxaliplatin Resistance in Colorectal Cancer by Promoting EZH2 Stability

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Research

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Abstract

Background Resistance to chemotherapy remains the major cause of treatment failure in patients with colorectal cancer (CRC). TRIM25, an E3-ubiquitin ligase, has been reported to play a vital role in tumorigenesis. This project aims to explore the function and mechanism of TRIM25 in regulating oxaliplatin resistance in colorectal cancer.

Methods The expression of TRIM25 in colorectal cancer tissues were examined by publicly available dataset, Immunohistochemistry and western blot. Further survival analysis was conducted using Kaplan-Meier method. CCK8 assay, colony-formation assay, Annexin V-FITC /PI staining and xenograft tumor models were used for evaluating sensitivity of CRC cells to oxaliplatin. Sphere-formation assay, RT-PCR and limiting dilution assay were used to evaluate the influence of TRIM25 on stem cell properties of CRC cells. Co-immunoprecipitation, polyubiquitination assay and western blot elucidate the mechanism by which TRIM25 regulates EZH2.

Results Patients with high expression of TRIM25 have significantly higher recurrence rate (28.9% vs. 15.0%, $P = 0.012$) and worse disease-free survival ($P = 0.006$) than those with low TRIM25 expression. Downregulation of TRIM25 dramatically inhibited while TRIM25 overexpression enhanced CRC cells survival after oxaliplatin treatment. In addition, TRIM25 promotes stem cell properties of CRC cells both in vitro and in vivo (8 mice per group). Importantly, we demonstrated that TRIM25 inhibits the binding of E3-ubiquitin ligase TRAF6 to EZH2, thus stabilizing and upregulating EZH2 and promoting oxaliplatin resistance.

Conclusions Our study provides evidence that TRIM25 is a novel epigenetic regulator of oxaliplatin resistance. Targeting TRIM25 might be a promising strategy for CRC treatment.

Background

Colorectal cancer (CRC) is the third most common cancer throughout the world and the fourth leading cause of cancer-related mortality [1]. Surgical resection plus oxaliplatin (OXA)-based chemotherapy is the most frequently used therapeutic strategy for CRC patients [2]. However, cancer cells eventually develop chemoresistance, which is considered the major cause of treatment failure in patients with metastatic cancer and a major limitation of the efficacy of chemotherapy drugs in clinical practice [3]. Thus, revealing the underlying mechanism and discovering new therapeutic targets are necessary to improve treatment outcomes for CRC patients.

Cancer stem cells (CSCs) are a group of special cellular sub-populations, which exhibit self-renewing and tumorigenic capacity [4, 5]. Additionally, CSCs express multidrug resistance ATP-binding cassette (ABC) transporters and DNA repair enzymes, which protect CSCs from drug-induced apoptosis [6]. In recent years, emerging evidence has suggested that the presence of CSCs is a key factor in tumor resistance to chemotherapeutic drugs [7]. Therefore, triggering CSCs to exit the stem-like state and as a result increasing the response of cancer cells to chemotherapy is a novel means of overcoming chemotherapy

resistance. Epigenetic regulation of transcriptional programs, including DNA methylation, non-coding RNAs and histone posttranslational modifications (acetylation, methylation, etc.), is a key driver of self-renewal capacity [8-10]. Enhancer of zeste homolog 2 (EZH2) is a critical component in epigenetic polycomb repressive complex 2 (PRC2) and silences target genes via tri-methylating histone H3 on lysine 27 (H3K27me3) [11]. Up-regulation of the H3K27me3 methyl-transferase EZH2 has been observed in various cancers. It has been shown that EZH2 is associated with a high proliferation rate, aggressive tumor subtypes, and poor outcome of cancer patients [12]. Besides, EZH2 is reported to contribute to chemotherapy response, and EZH2 inhibitors have been proved to reverse drug-resistance in cancers [13, 14]. Recent studies showed that EZH2 plays an essential role in maintaining CSC properties in multiple cancer types including breast cancer, prostate cancer and glioblastoma [15-17]. In the context of CRC, EZH2 has been proved to contribute to the CSC state by modulating key pathways such as Wnt/ β -catenin and Hedgehog signaling, where high activity has been shown to designate the CSC population [14]. Therefore, EZH2 might be a promising target for cancer therapy.

TRIM25 is a member of the tripartite motif (TRIM) family that functions in multiple RNA-dependent pathways [18]. Like a typical TRIM protein, TRIM25 consists of an N-terminal tripartite motif or RBCC motif and a C-terminal SPRY domain [19-21]. There is accumulating evidence suggesting TRIM25 plays a key role in many physiological disorders, predominantly by regulating ubiquitination of the target protein. For example, TRIM25 is crucially involved in interferon signaling through mediating K63-linked polyubiquitination of RIG-1 [20]. In addition to modulating cellular antiviral activity, TRIM25 also participates in tumorigenesis. High expression of TRIM25 has been demonstrated in a variety of cancers such as CRC, lung cancer, and breast cancer [22-24]. In hepatocellular carcinoma, TRIM25 promotes cancer cell survival and growth through targeting Keap1-Nrf2 pathway [25]. Despite extensive research on TRIM25 in cancer, the role of TRIM25 in regulating drug-resistance remains largely unknown.

In this study we revealed the previously unknown mechanism of oxaliplatin chemoresistance. We demonstrated that the histone H3K27me3 methyl-transferase EZH2 expression is regulated by TRIM25 in CRC cells. TRIM25 inhibits the binding of TRAF6, an E3 ubiquitin ligase, to EZH2, and stabilizes EZH2 to promote oxaliplatin resistance. Our findings suggest that TRIM25 is a novel epigenetic regulator, and targeting TRIM25–EZH2 pathway may be a promising approach for CRC treatment.

Materials And Methods

Cell lines and cell culture

The CRC cell lines (SW48 and SW480) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured under conditions as recommended. All cell lines were authenticated using short tandem repeat (STR) fingerprinting and negatively tested for mycoplasma contamination before experiments.

Patients and tissue specimens

The formalin-fixed, paraffin-embedded CRC tissues (n = 223) were collected from surgical specimens between November 2007 and December 2012 at the Sun Yat-sen University Cancer Center. Patient characteristics were summarized in Supplementary Table S1. All patients treated with a FOLFOX or XELOX regimen and were followed up with regular surveillance at our hospital. Approvals from the ethical committee of Sun Yat-sen University Cancer Center and prior patient's consents were previously obtained for the use of these clinical specimens for research purpose.

Immunohistochemistry

IHC analysis using paraffin-embedded CRC specimens was conducted following standard manufacturer's protocols as described previously. Primary antibody TRIM25 (Proteintech, 67314-1-Ig) and EZH2 (Cell Signaling Technology, #5246) were used for IHC staining. IHC staining was evaluated by two independent gastrointestinal pathologists blinded to the patients' characteristics and clinical outcome. Final IHC score was calculated based on both the extent and the intensity of staining. The staining extent that scored according to the percentage of positively stained cells ranged from 0 to 3 (0, 0-25%; 1, 25-50%; 2, 50-75%; and 3, 75-100%), while the intensity of staining was scored as 0 (negative staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). Specimens with the final score ≥ 4 were defined as high expression, and specimens with the final score < 4 were defined as low expression.

RNA interference

For knockdown of TRIM25, negative control (shNC) or target gene shRNAs were co-transfected into HEK293T with pHelper and pEnv. The virus was harvested from the transfected HEK293T cells, and target cells were infected with viral supernatants for 2 consecutive days. Stable CRC cell lines were then selected by treating with puromycin (2 $\mu\text{g/mL}$) for 10 days. Target sequences for shRNAs were shown in Supplementary Table S2.

Cell viability assay

CRC cells (5×10^3 cells/well) were seeded in 96-well plates and treated with corresponding processes. At the indicated time point, CCK8 was added into the wells and incubated with cells according to the product manual. Then the Thermomax microplate reader was used to measure the absorbance of each well at wavelength of 450 nm (A450).

Apoptosis assay

For cell apoptosis assay, colorectal cancer cells treated with oxaplatin were harvested and stained with Annexin V-FITC and propidium iodide (PI) using an Annexin V-FITC/PI-staining kit (BD Pharmingen™, San Diego, CA, USA). After incubated at room temperature for 15 minutes, the cells were analyzed by flow cytometry.

Immunoprecipitation (IP) and Ubiquitination analysis

Cancer cells were washed with cold PBS and lysed in NP-40 lysis buffer at 4 °C. For EZH2 ubiquitination detection, 24 hours after transfection, cells were treated with the proteasome inhibitor MG132 (10 μM) for 6 hours and lysed in NP-40 lysis buffer supplemented with protease-inhibitor cocktail. Total cell lysates were incubated with appropriate primary antibody overnight and subsequently rotated with protein A/G beads for 2~4 h at 4 °C. Beads were then washed with NP-40 lysis buffer for three times, mixed with 2 × SDS sample buffer and boiled for 10 min. The co-precipitates were analyzed by immunoblot analysis using a chemiluminescence method.

Xenograft tumor model

Male BABL/c nude mice (4–5 weeks old) were used to evaluate the clinical benefits of targeting TRIM25. For limiting dilution assay, indicated number of SW480 cells transfected with/without TRIM25 or shTRIM25 was subcutaneously injected to the left flank of each nude mouse. For tumors treated with oxaliplatin, TRIM25 overexpressing SW480 cells (5×10^5) treated with or without EZH2 inhibition were suspended in 100 μl PBS and subcutaneously injected to the left flank of each nude mouse. One week later, the mice were administered oxaliplatin (5 mg/kg twice a week) intraperitoneally. Tumor growth curve was measured for 3 weeks and the volume of tumor was calculated as $\text{Length} \times \text{Width}^2 \times 1/2$. At the end of the study, we surgically removed the tumors from sacrificed mice. The animal experiments were conducted according to the Animal Study Guidelines of the Ethics Committee of Sun Yat-Sen University.

Statistical analysis

Statistical analyses were performed using SPSS version 24.0 software. All data were presented as mean ± SD. Statistical tests used in this study included the two-tailed Student's t test, χ^2 test and log-rank test. *P*-value < 0.05 was considered statistically significant.

Results

High expression of TRIM25 predicts recurrence in patients with CRC

We analyzed publicly available CRC mRNA expression profiles (GSE20842) obtained from the NCBI and found that the mRNA expression of TRIM25 is elevated in CRC tissues compared with normal tissues (Fig. 1A). To confirm our *in silico* observations, we detected TRIM25 protein expression in CRC samples. Immunohistochemistry (IHC) analysis demonstrated that TRIM25 protein level in CRC tissues was significantly higher than that in adjacent normal tissues (Fig. 1B and C). To explore the potential role of TRIM25 in CRC therapy, we initially evaluated TRIM25 expression in 26 primary tumor tissues from patients with stage III CRC that were treated with oxaliplatin-based chemotherapy. The results showed that TRIM25 protein level was significantly higher in oxaliplatin resistant patients who developed recurrence during follow-up period (Fig. 1D). We further analyzed TRIM25 expression in 223 paraffin-embedded human CRC specimens from patients with Xelox or FOLFOX treatment after surgery (patient characteristics were summarized in Supplementary Table 1). Representative IHC staining confirmed that

TRIM25 expression was markedly increased in CRC patients with tumor relapse (Fig. 1E). Patients with high TRIM25 expression demonstrated an observably higher recurrence rate than those with low TRIM25 expression (28.9% vs. 15.0%, $P = 0.012$, Fig. 1F and Table 1). Furthermore, compared with low TRIM25 expression, high TRIM25 expression was significantly associated with worse overall survival and disease-free survival ($P = 0.006$, Fig. 1G and Supplementary Figure S1). Collectively, the above observations suggest that elevated expression of TRIM25 contributes to the progression of CRC and is associated with the failure of OXA-based chemotherapy.

TRIM25 confers OXA resistance in CRC cells in vitro

We then examined whether TRIM25 was, in fact, associated with resistance to OXA-based therapy in preclinical models. Firstly, we established stable TRIM25 knockdown and overexpressing cells from the SW48 and SW480 CRC cell lines (Fig. 2A). The CCK8 assay showed that the IC50 values for OXA were decreased in TRIM25-knockdown cells and were increased in TRIM25-overexpressing cells (Fig. 2B and C). Consistently, in the presence of OXA, TRIM25 knockdown dramatically inhibited while TRIM25 overexpression enhanced the colony-formation ability of SW48 and SW480 cells (Fig. 2D). Moreover, compared with control cells, knockdown of TRIM25 resulted in significantly increased OXA-induced apoptosis of CRC cells, while overexpression of TRIM25 reduced OXA-induced apoptosis (Fig. 2E). Measurement of cleaved caspase 3 and cleaved PARP further confirmed that knockdown of TRIM25 increased OXA sensitivity and upregulation of TRIM25 conferred OXA resistance (Fig. 2F). Altogether, these results reveal that TRIM25 confers OXA resistance in CRC cells.

TRIM25 promotes stem cell properties of CRC cells

As reported previously, stemness is believed to be responsible for chemotherapy resistance, thus we hypothesized that TRIM25 is involved in regulating CRC stemness. A couple of experiments were then performed to test our hypothesis. We conducted sphere formation assay, and found a decrease in sphere numbers and sizes in TRIM25 knockdown cells compared with the corresponding control cells; while overexpression of TRIM25 enhanced the sphere formation ability of CRC cells (Fig. 3A). In addition, the expression of stem cell related molecules such as EpCAM, SOX2, CD133 and CD44 in SW48 and SW480 cells was markedly reduced after TRIM25 inhibition, while the expression increased after TRIM25 overexpression (Fig. 3B). Furthermore, limiting dilution analysis (LDA) in vivo confirmed the markedly reduced stem cell frequency in TRIM25 knockdown SW480 cells (Fig. 3C), with forming smaller and lighter tumors than the control SW480 cells (Fig. 3D and E). And no visible tumors could be formed in nude mice when 1×10^4 TRIM25 knockdown SW480 cells were inoculated. These findings indicate the crucial role of TRIM25 in promoting stem cell properties of CRC cells.

TRIM25 regulates EZH2 stability in CRC cells

As the catalytic subunit of PRC2, EZH2 plays an essential role in tumor progression. Previous study has revealed that targeting EZH2 inhibits CSC self-renewal and enhances the sensitivity of CRC to OXA [13-14]. In the present study, we found that knockdown of TRIM25 decreased the protein level of EZH2 (Fig.

4A). However, there was no significant effect on EZH2 mRNA levels in both SW48 and SW480 cells (Fig. 4B). These results suggested that TRIM25 may affect the stability of EZH2. To substantiate this assumption, we treated TRIM25-knockdown or control CRC cells with the protein synthesis inhibitor cycloheximide (CHX) and the proteasome inhibitor MG132. The results showed that knockdown of TRIM25 shortened the half-life of endogenous EZH2 protein in CRC cells after CHX treatment (Fig. 4C and D). Besides, the expression of EZH2 was modestly increased in TRIM25 knockdown SW48 cells treated with MG132, and the same results were obtained in SW480 cells (Fig. 4E). Finally, we detected EZH2 protein in the same cohort of CRC samples by IHC staining, and found that EZH2 high expression was significantly positively correlated with TRIM25 high expression (Fig. 4F). CRC samples with high TRIM25 expression showed a higher rate of high EZH2 expression, whereas samples with low TRIM25 expression exhibited lower rate of high EZH2 expression (63.9% vs. 45.7%, $P = 0.009$, Fig. 4G). CRC patients with high expression of TRIM25 as well as high expression of EZH2 had the shortest overall survival ($P 0.001$, Supplementary Figure S2) and disease-free survival ($P 0.001$, Fig. 4H) compared with patients with low TRIM25 expression or low EZH2 expression. Taken together, these observations suggest that TRIM25 regulates EZH2 expression in CRC cells through reducing degradation of EZH2 protein.

TRIM25 blocks TRAF6-mediated ubiquitination of EZH2

Since TRIM25 is an E3 ligase, we wondered if TRIM25 modulates EZH2 stability through the ubiquitin–proteasome pathway. First, we analyzed the interaction of TRIM25 with EZH2 in CRC cells. By co-immunoprecipitation and western blot, we verified the interaction between TRIM25 and EZH2 at both exogenous and endogenous protein levels (Fig. 5A and B). Then, we performed *in vivo* ubiquitination assays in HEK293T cells transfected with siTRIM25, Flag-EZH2, His-tagged ubiquitin wild type (WT) or mutation plasmids (K48 or K63 mutants). As shown in Fig. 5c, knockdown of TRIM25 increased the poly-ubiquitination of EZH2, indicating that EZH2 is not a substrate of TRIM25 E3 ligase. Interestingly, EZH2 polyubiquitination enhanced by knockdown of TRIM25 was mainly extended through K63-linkage instead of K48-linkage.

TNF receptor associated factor 6 (TRAF6) is a member of the TNF receptor associated factor (TRAF) protein family, and functions as an E3 ubiquitin ligase and a scaffold protein. It has been reported that TRAF6 mediated the K63-linked ubiquitination of EZH2 in prostate cancer [26]. We then speculated whether TRIM25 is involved in TRAF6-mediated ubiquitination of EZH2. We transfected siNC and siTRIM25 in SW480 cells, and the expression of TRAF6 was detected in EZH2 immunoprecipitates. We interestingly found that knockdown of TRIM25 enhanced the interaction between EZH2 and TRAF6 (Fig. 5D). To further confirm the role of TRIM25 in TRAF6-mediated EZH2 ubiquitination, we blocked TRAF6 in TRIM25 knockdown SW480 cells. Further co-immunoprecipitation and immunoblotting analysis showed that knockdown TRIM25-induced EZH2 ubiquitination and degradation could be rescued by siTRAF6 (Fig. 5E and F). Taken together, these results suggest that TRIM25 stabilizes EZH2 most probably by preventing TRAF6 bind to EZH2.

EZH2 is required for TRIM25-induced OXA-resistance in CRC both in *vitro* and in *vivo*

To further assess whether TRIM25 mediates OXA-resistance via EZH2, we blocked EZH2 in TRIM25-overexpressing cells by shRNA or EZH2 inhibitor (UNC1999). Colony formation assay, CCK8 assay and Annexin V/PI apoptosis assay showed that inhibition of EZH2 significantly rescues the effect of TRIM25 overexpression on both the growth and anti-apoptotic capacity of CRC cells treated with OXA (Fig. 6A, B and C). Besides, the sphere formation ability of SW48 and SW480 cells overexpressing TRIM25 was markedly suppressed by EZH2 inhibition (Fig. 6D).

Moreover, nude mouse xenograft model was used to evaluate the effect of EZH2 inhibition on TRIM25-induced OXA-resistance *in vivo*. Consistent with *in vitro* findings, the results showed that inhibition of EZH2 by shEZH2 or UNC1999 resulted in a significant reduction in tumor volume and weight when treated with OXA (Fig. 6E and F). And further TUNEL staining showed that inhibition of EZH2 resulted in more OXA-induced apoptosis by comparison with TRIM25-overexpressing cells *in vivo*, as demonstrated by a higher proportion of TUNEL positively stained cells after OXA treatment (Fig. 6G and H). Overall, EZH2 is essential for TRIM25-induced OXA-resistance in CRC, and inhibition of EZH2 is expected to overcome TRIM25-induced OXA-resistance in clinical practice.

Discussion

Oxaliplatin is one of the most common chemotherapeutic agents used for the treatment of CRC. However, the toxicity to normal cells and chemotherapy resistance are two major barriers to satisfactory tumor regression [27–29]. CSCs are a subpopulation of tumorigenic cells that are naturally resistant to chemotherapy, due to the aberrant DNA repair ability and multidrug resistance ABC transporters expression [30]. Therefore, targeting CSCs might be a promising approach for CRC treatment.

TRIM proteins, which represent the largest class of RING-containing E3 ubiquitin-ligases, were involved in diverse cellular processes, such as innate immunity and tumorigenesis [19, 25]. In the present study, we demonstrated that TRIM25 was highly expressed in CRC tissues, and high expression of TRIM25 was significantly associated with tumor relapse in primary CRC patients, who received OXA-based therapy after surgical resection. As an E3-ubiquitin ligase, TRIM25 is known to regulate the ubiquitination of various proteins. It has been shown that TRIM25 mediates K63-linked polyubiquitination of the amino-terminal caspase recruitment domains (CARDs) of RIG-I, which is critical for its activation [20]. Besides, Liu et al. reported that TRIM25 directly targets Keap1 by ubiquitination to activate Nrf2 signaling pathway in liver cancer [25]. Here, we found a positive association between TRIM25 and EZH2 expression, suggesting that EZH2 may be a major target of TRIM25 in CRC. Indeed, TRIM25 stabilizes EZH2 protein, and subsequently enhances CSC properties and confers the resistance of CRC to OXA-based treatment. These findings suggest that TRIM25 may serve as a potential therapeutic target for enhancing OXA response of CRC patients.

Previous studies have identified the driving role of EZH2 in stem cell self-renewal. In CRC, EZH2 functions as a transcriptional repressor to downregulate IHH, a key gene responsible for normal colonocyte differentiation, resulting in improvement of self-renewal capacity of CSC [14]; in breast cancer, EZH2

increases NOTCH1 expression by directly binding to the NOTCH1 promoter and further promotes CSC properties or expands CSCs [16]. Besides its canonical function via regulation of H3K27me3, EZH2 is reported to increase the self-renewal capacity of CSC through binding to some non-histone targets, such as STAT3, NF- κ B and β -catenin [31–33]. Therefore, targeting EZH2 is thought to be a rational and innovative strategy for the treatment of CRC. Despite the established role in CSC, the regulation of EZH2 is not fully understood. Posttranslational modifications such as phosphorylation, glycosylation and ubiquitination, are thought to be the main factors affecting the stability and activity of EZH2. For example, phosphorylation at Serine 21 mediated by AKT suppresses the activity of EZH2, thereby decreasing H3K27me3 levels [34]; glycosylation of EZH2 is required for its stability and enzymatic activity [35, 36]. The regulation of ubiquitination of EZH2 is complex, and several E3 ligases, such as Smurf2, β -TrCP, Praja1 and FOXP3, have been reported to be involved in these processes [37–40]. TRAF6 is a kind of adaptor protein and E3 ubiquitin ligase that belongs to the family of tumor necrosis factor receptor-associated factors (TRAFs), and plays a vital role in various solid tumors through activating multiple signaling pathways [41, 42]. Recent study showed that TRAF6 could catalyze K63-linked polyubiquitination of EZH2 and promote its proteasome-dependent degradation in prostate cancer [26].

Interestingly, our present results show that silencing TRIM25 promotes the ubiquitination of K63-linked polyubiquitin chains on EZH2 and decreased its half-life. Our results further indicate that TRAF6 is crucial for the ubiquitination of EZH2 regulated by TRIM25. This regulation could be explained by two possible mechanisms. One possibility is that TRIM25 directly inhibits the catalytic activity of TRAF6. The E3 ubiquitin ligase activity of TRAF6 requires autoubiquitinated through Lys63 (K63)-linked ubiquitin chains, which depends on its ring finger domain. Indeed, Lee et al. has reported that deletion of TRIM25 inhibits TRAF6 ubiquitination and TRAF6 mediated NK- κ B activation [43]. The second possible mechanism is that TRIM25 may compete with TRAF6 to bind EZH2. This is supported by our data in the present study, showing that knockdown of TRIM25 promoted the interaction between TRAF6 and EZH2. These two mechanisms are not mutually exclusive, as suppression of TRAF6 activity may inhibit the binding of EZH2 to TRAF6. This is consistent with previous reports that TRIM proteins can bind a protein to prevent its ubiquitination by another E3-ubiquitin ligase. For example, TRIM17 binds to BCL2A1 and prevents TRIM28-mediated ubiquitination and degradation of BCL2A1 in melanoma cells [44]. Besides, TRIM24 is shown to inhibit the degradation of dysbindin in cardiomyocytes in a similar manner [45].

Conclusions

This study described the role of TRIM25 in mediating OXA resistance and a new pathway for regulating EZH2 stability in CRC. The mechanisms underlying the acquisition of OXA resistance have yet to be fully characterized, but we provide evidence that TRIM25-dependent EZH2 upregulation is associated with this process. Moreover, we demonstrated that TRIM25 may be a valuable biomarker for the identification of CRC patients who are likely to respond favorably to OXA treatment. This new information therefore suggests that developing inhibitors against this function of TRIM25 might be an excellent strategy for CRC management.

Abbreviations

CRC

Colorectal cancer

CSC

Cancer stem cell

EZH2

Enhancer of zeste homolog 2

IHC

Immunohistochemistry

OXA

Oxaliplatin

CHX

Cycloheximide

RT-PCR

Real-time transcription-polymerase chain reaction

IP

Immunoprecipitation

Ub

Ubiquitin

Declarations

Availability of data and materials

All data supporting our findings are included in the paper and its supplemental information files.

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Author contributions

ZLH, ZZP and SZ designed the study. SZ and JHP contributed to the data acquisition and analysis. LNX constructed the expression plasmids and analyzed the data. YJF, QJO and JYQ analyzed public databases and participated in animal experiments. MZL and CXZ contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Approvals from the ethical committee of Sun Yat-sen University Cancer Center and prior patient's consents were previously obtained for the use of these clinical specimens for research purpose.

The animal experiments were conducted according to the Animal Study Guidelines of the Ethics Committee of Sun Yat-Sen University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1
Association of TRIM25 expression with patient characteristics.

Characteristic	TRIM25 Expression		P-value
	Low	High	
Age (years)			0.245
<56	72 (51.4%)	36 (43.4%)	
≥56	68 (48.6%)	47 (56.6%)	
Sex			0.332
Male	75 (53.6%)	50 (60.2%)	
Female	65 (46.4%)	33 (39.8%)	
Tumor location			0.914
Right-sided	55 (39.3%)	32 (38.6%)	
Left-sided	85 (60.7%)	51 (61.4%)	
Primary tumor size			0.668
<4.5 cm	75 (53.6%)	42 (50.6%)	
≥4.5 cm	65(46.4%)	41 (49.4%)	
T stage			0.012
T1-2	66 (47.1%)	25 (30.1%)	
T3-4	74 (52.9%)	58 (69.9%)	
N stage			0.522
N1	92 (65.7%)	58 (69.9%)	
N2	48 (34.3%)	25 (30.1%)	
TNM stage			0.059
T1-3N1M0	47 (33.6%)	18 (21.7%)	
T4NanyM0 or TanyN2M0	93 (66.4%)	65 (78.3%)	
Recurrence			0.012
Yes	21 (15.0%)	24 (28.9%)	
No	119 (85.0%)	59 (71.1%)	

Figures

Figure 1

High expression of TRIM25 is associated with recurrence in colorectal cancer. (A) Analysis of TRIM25 expression in CRC and adjacent normal tissues from publicly available GEO dataset (GSE20842), t-test. (B) Representative IHC images of TRIM25 in colorectal tumor or normal tissues. Scale bar, 200µm. (C) IHC score of TRIM25 expression in paired normal and CRC samples, paired t-test. (D) Western blot analysis showing the expression of TRIM25 in 26 primary tumor tissues from patients with stage III CRC that were treated with oxaliplatin-based chemotherapy, including 13 samples developed recurrence and 13 samples did not develop recurrence after surgical resection. (E) Representative IHC images of TRIM25 in 223 CRC tissues with or without recurrence. Scale bar, 200µm. (F) Relationship between TRIM25 expression and CRC recurrence, χ^2 test. (G) Comparison of disease-free survival between patients with high or low TRIM25 expression, log-rank test.

Figure 2

TRIM25 influences the sensitivity of CRC cells to oxaliplatin. (A) Western blot analysis of TRIM25 in SW48 and SW480 cells transfected with shTRIM25 or TRIM25-expressing plasmid. GAPDH was used as the loading control. (B-C) CCK8 assay showing the IC₅₀ values for OXA in TRIM25-knockdown and TRIM25-overexpressing SW48 and SW480 cells. (D) Colony-formation ability (left panel: representative images; right panel: quantification of colony number) of the indicated cells treated with oxaliplatin. (E)

Annexin V-FITC and PI staining showing OXA-induced apoptosis in the indicated CRC cells. Left panel: representative images; right panel: quantification of apoptotic cells. (F) Western blot analysis of cleaved caspase 3 and cleaved PARP in the indicated cells treated with or without OXA. Data represent as Mean $\pm$ SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3

TRIM25 promotes stem cell properties of CRC cells. (A) Representative images (left panel) and quantification (right panel) of the in vitro sphere-formation assay of TRIM25-knockdown and TRIM25-overexpressing SW48 and SW480 cells. Scale bar, 200 μ m. Data represent as Mean $\pm$ SD of three independent experiments. (B) Relative mRNA expression of EpCAM, SOX2, CD133 and CD44 in TRIM25-knockdown or TRIM25-overexpressing SW48 and SW480 cells. Data represent as Mean $\pm$ SD of three independent experiments. (C) Limiting dilution data showing the stem cell frequency of the indicated group of SW480 cells from a tumorigenic mouse model. (D) Growth curves of each group of tumors. Indicated numbers of SW480 cells were inoculated into the nude mice and tumor volumes were measured every four days. Data represent as Mean $\pm$ SD. (E) Each group of tumors were removed and weighted. Data represent as Mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001 for indicated comparison.

Figure 4

TRIM25 regulates EZH2 stability in CRC cells. (A) Western blot analysis of EZH2 protein in control and TRIM25 knockdown SW48 and SW480 cells. (B) RT-PCR analysis of EZH2 mRNA in control and TRIM25 knockdown SW48 and SW480 cells. (C) EZH2 protein levels in control and TRIM25 knockdown SW48 or SW480 cells were detected at the indicated time points after CHX (20 μ g/mL) treatment. (D) And relative level of EZH2 protein at each time point was normalized by GAPDH. (E) Western blot showing the effect

of MG132 on EZH2 protein levels in control and TRIM25 knockdown SW48 or SW480 cells. (F) Representative IHC images of TRIM25 and EZH2 in two CRC cases. Scale bar, 100µm. (G) Relationship between TRIM25 expression and EZH2 expression in the same cohort of CRC samples, χ^2 test. (H) Comparison of disease-free survival between patients with different TRIM25 and EZH2 expression patterns, log-rank test.

Figure 5

TRIM25 blocks TRAF6-mediated ubiquitination of EZH2. (A) Endogenous interaction between TRIM25 and EZH2 determined by co-immunoprecipitation with anti-TRIM25 or anti-EZH2 antibody in SW480 cells. (B) Exogenous interaction between TRIM25 and EZH2 determined by co-immunoprecipitation with anti-Flag or anti-HA antibody in HEK293T cells co-transfected with Flag-EZH2 and HA-TRIM25. (C) Flag-EZH2, siTRIM25, His-tagged ubiquitin wild type (WT) or mutation plasmids (K48 or K63 mutants) were transfected into HEK293T cells as the indicated combinations. Immunoprecipitation with anti-Flag was performed to detect the ubiquitination level of EZH2. (D) Immunoprecipitation analyses using IgG and anti-EZH2 in SW480 cells transfected with siNC or siTRIM25, followed by western blot with the indicated antibodies. (E) Flag-EZH2, siTRIM25, siTRAF6, His-tagged ubiquitin wild type (WT) were transfected into HEK293T cells as the indicated combinations. Immunoprecipitation with anti-Flag and western blot with anti-His were performed to detect the ubiquitination level of EZH2 under different conditions. (F) Western blot analysis of EZH2 protein in SW480 cells transfected with siTRIM25 and/or siTRAF6.

Figure 6

EZH2 is required for TRIM25-induced OXA-resistance in CRC. (A) Colony-formation ability of the indicated cells treated with oxaliplatin. Left panel: representative images; right panel: quantification of colony number. (B) CCK8 assay showing the IC₅₀ values for OXA in the indicated cells. (C) Annexin V-FITC and PI staining showing OXA-induced apoptosis in the indicated CRC cells. Left panel: representative images; right panel: quantification of apoptotic cells. (D) Representative images (left panel) and quantification

(right panel) of the in vitro sphere-formation assay using the indicated CRC cells. Scale bar, 200µm. (E) Growth curves and tumor volume of xenograft tumors derived from TRIM25-overexpressing SW480 cells treated with control (PBS), shEZH2 or UNC1999 (n = 6 mice for each group). (F) Tumor weight of each group of xenograft tumors. (G) Apoptotic cells in paraffin-embedded tumor sections derived from each group of SW480 cells bearing mice were visualized by TUNEL staining. (H) Quantification of TUNEL positively stained cells in the indicated group of tumor sections (n = 6). Data represent as Mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 for indicated comparison.

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