TIFA Promotes CRC Cell Proliferation via RSK- and PRAS40- Dependent Manner

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Abstract

Background: Previous studies have shown that TIFA (TNF receptor associated factor (TRAF)-interacting protein with a Forkhead-associated (FHA) domain) plays different roles in various tumor types. However, the function of TIFA in colorectal cancer (CRC) remains unclear. The goal of this study is to uncover the biological function and molecular mechanism of TIFA in CRC.

Methods: Tissue microarrays were used to evaluate TIFA expression. Cancer cell proliferation assays were performed in TIFA knockdown and overexpressing cells *in vitro* and in a xenograft model *in vivo*. Human phosphokinase array, immunoprecipitation assays were performed to explore the underlying mechanism.

Results: We disclosed that the expression of TIFA was markedly increased in CRC versus normal tissue, and positively correlated with CRC TNM stages. In agreement, we found that the CRC cell lines show increased TIFA expression levels versus normal control. The knockdown of TIFA inhibited cell proliferation but have no effects on cell apoptosis *in vitro* and *in vivo*. Moreover, the ectopic expression of TIFA enhanced cell proliferation ability *in vitro* and *in vivo*. In contrast, the expression of mutant TIFA (T9A, oligomerization site mutation; D6, TRAF6 binding site deletion) alternatively abolished TIFA mediated cell proliferation enhancement. Exploration of the underlying mechanism demonstrated that the protein synthesis associated kinase RSK and PRAS40 activation were all responsible for TIFA mediated CRC progression.

Conclusions: The above results described a model of TIFA in mediating CRC progression. This may provide a promising target for CRC therapy.

Introduction

As one of the common human malignancies, colorectal cancer (CRC) has higher morbidity and mortality which is ranked as the third most common diagnosed cancer in males and the second in females, respectively [1]. More than 1.4 million new cases and nearly 0.7 million death of CRC per year globally was reported [2]. With the significant progress in screening and treatment strategies has occurred, the prognosis of CRC has steadily improved, and the relative survival rate of 5-year is largely improved [3]. However, effective strategies targeting tumor metastasis, chemo-resistance or recurrence to prevent CRC progress remain lacking. Based on this, developing novel new prognosis biomarkers or targets to predict the CRC prognosis, to perform CRC targeted therapies is in urgent need.

TIFA (TRAF-interacting protein with a Forkhead-associated domain), first named as T2BP [4], was identified as a novel TRAF6 interacting protein by using yeast two-hybrid screening [5]. In the structure of TIFA, it contains a FHA domain which could directly bind phosphothreonine/phosphoserine, and a consensus TRAF6-binding motif which could interact with TRAF6. In the function of TIFA, TIFA oligomerization which induced oligomerization and poly-ubiquitinylation of TRAF6, further in turn activates TAK and IKK [6], finally mediates NF- κ B activation in the TLR4/IL-1 signaling pathway [7]. Recent researches also offered an insight into the role of TIFA for innate immunity induced by bacterial

metabolite HBP (heptose- 1,7-bisphosphate) or NLRP3 inflammasome [8–10]. Moreover, it is also proved that TIFA promotes cancer cell migration, and cell survival in lung adenocarcinoma [11]. Our previous work indicated that TIFA expression is down-regulated in HCC progression and TIFA reconstitution promotes HCC cell apoptosis, while suppressing HCC cell proliferation among surviving cells [12, 13]. The above previous findings implicate TIFA plays various roles in different cancer types. However, the function of TIFA in colorectal cancer (CRC) was not dissected.

Cell cycling and protein synthesis both play irreplaceable role in cancer cell proliferation [14]. Protein synthesis is also a key step which occurred in G0/G1 and G2/M phase of cell cycle. RSK (Ribosomal S6 protein kinase) is a family of serine/threonine protein kinases comprises four main members (RSK1, RSK2, RSK3 and RSK4), and they could activate downstream of the mitogen-activated protein kinase (MAPK) pathway [15]. RSK has multiple functions of RSK were found which contains the involvement in cancer cell proliferation, invasion and migration [16–19]. RSK was proved to regulate the stability of eIF (eukaryotic translation initiation factor) to mediate protein synthesis [20, 21]. It was also reported that RSK could combine with TRAF6 and activate each other [22]. Nevertheless, the specific function of RSK in TIFA mediated cancer progression remains unreported.

PRAS40 (Proline-Rich Akt Substrate of 40 kDa) also called 14-3-3 binding protein, was first identified in insulin-treated hepatoma cells lysates [23]. Further studies of PRAS40 found that it was a component and substrate of the mTOR complex 1 (mTORC1) [24, 25]. PRAS40 phosphorylation by the kinases like AKT or by mTORC1 itself could induce the dissociation of PRAS40 from mTORC1 and relieve an inhibitory constraint on mTORC1 activity [26]. But PRAS40 is also indispensable for the activity of the mTORC1 complex [27]. PRAS40 was reported to high-expressed in cancer and mediated cancer progression via activating mTOR signaling [28–30]. But the role of PRAS40 in TIFA mediated cancer progression remains unrevealed.

In the present study, we investigated the specific function and mechanism of TIFA in promoting the CRC progression *in vitro* and *in vivo*. We demonstrated that the expression of TIFA was marked increased in CRC tissue and cell lines versus normal control. The knockdown of TIFA inhibited CRC cell proliferation but have no effects on cell apoptosis *in vitro* and *in vivo*. Moreover, the ectopic expression of TIFA enhanced cell proliferation ability *in vitro* and *in vivo*. In contrast, the expression of mutant TIFA (T9A, oligomerization site mutation; D6, TRAF6 binding site deletion) alternatively abolished TIFA mediated cell proliferation enhancement. Exploration of the underlying mechanism demonstrated that the protein synthesis associated kinase RSK and PRAS40 activation were all responsible for TIFA mediated CRC progression. In summary, the aforementioned findings suggest a model of TIFA in mediating CRC progression. This may provide a promising target for CRC therapy.

Materials And Methods

Tumor tissues

The CRC tissues we used were obtained from the patients who underwent surgical treatment at the Affiliated Hospital of Jining Medical University from 2015 to 2019. All tissues from the patients were staged based on the American Joint Committee on Cancer TNM staging system. This research was approved by the institutional ethics committees at the Jining Medical University.

Cell culture

SW620 colon cancer cells were cultured in L15 medium supplemented with 10% FBS (Gibco). RKO cancer cells were cultured in DMEM medium supplemented with 10% FBS, 1% NEAA, 1% sodium pyruvate and 1.5g/L NaHCO₃. SW480 cancer cells were cultured in DMEM medium supplemented with 10% FBS and 2% L-Glutamine. RKO and SW480 cells were grown at 37°C in 5% CO₂ incubators and SW620 were grown at 37°C in 0% CO₂ incubators. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. All cells were purchased from ATCC and all were tested to ensure that they were Mycoplasma negative.

Vector Construction

ShRNA targeting human TIFA and human TIFA or mutant TIFA overexpression plasmid were constructed following the published protocol [12, 13]. The primers used in this assay were listed in **Supplementary Table 1**.

CCK8 assay

CCK-8 assay was performed on colon cancer cells following the previous protocols [31]. In the 96-well plate, 3×10^3 cells were seed per well (day 0) and incubated for the following 4 or 5 days. 10 µl of CCK8 reaction agent was added to each well at the time of cell collection, then the optical density (OD) was measured at 450 nm by multiscanner autoreader.

Cell clone formation

Colon cancer cells were seeded at a density of 500 cells/mL in 6-well plate, then cultured with complete medium for two weeks, the cell clones were treated with 4% paraformaldehyde fixation and stained by Giemsa (contain 1% Trion X-100) for several hours, the clone photos were taken by camera and the colony numbers were calculated by Image J software.

Flow cytometry analysis of apoptosis and cell cycle

For cell cycle assay, colon cancer cells were stained with propidium iodide (BD Biosciences). For cell apoptosis assay, apoptotic cells were stained with PI and Annexin-V-FITC (BD Biosciences). The details of these assay could follow the previous protocols that we published [12, 13].

Western blot

Western blot was performed according to the established protocols described previously [12, 13]. Primary antibodies used in this assay are listed in **Supplementary Table 2**. All western blot results are provided as representative images from three independent experiments.

Immunohistochemistry (IHC)

IHC assay was performed on human colorectal cancer tissue microarrays (Avilabio Company, cat. # col11042) or paraffin-embedded specimens from *in vivo* tumors. The specific antibodies used for targeting corresponding proteins which listed in **Supplemental Table 2** and scored based on the percentage of proteins-positive cells in each tissues. The photos were taken by panoramic viewer under a $\times 10$ or $\times 40$ objective (3DHistech, Hungary) [32].

Immunoprecipitation

Western blot was performed according to the established protocols described previously [32, 33].

Antibodies used in this assay are listed in **Supplementary Table S2**.

Human Phospho-Kinase Array Kit

The phosphor-kinase array was performed with human phosphor-kinase array kit (R&D system, Cat. # ARY003B) following the manufacturer's instruction.

Xenograft Model Study

The *in vivo* xenograft model studies were first approved by Jining Medical University Ethics Committee. Nude mice with 6–8 weeks age were randomly assigned to each group ($n \geq 6$). The stable cell lines (1×10^6 cells) were injected to each mouse subcutaneously. With the tumors were palpable, the tumors were calculated and recorded. The tumor volume was calculated by the following formula: volume (mm^3) = (width² (mm^2) $\times$ length (mm))/ 2. Finally the mice were sacrificed, the tumors were separated and with formalin fixed, paraffin embedded, and sectioned for further analysis [32].

Statistical analysis

All data were analyzed using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA). Values were expressed as means $\pm$ SEM. P values were calculated using a two-tailed Student's t-test (two groups) or one-way ANOVA (more than 2 groups) unless otherwise noted. A value of $P < 0.05$ was used as the criterion for statistical significance. *Indicates significant difference with $P < 0.05$, **indicates significant difference with $P < 0.01$, ***indicates significant difference with $P < 0.001$ [32, 34, 35].

Results

TIFA was up-regulated in human colorectal cancer tissues

To investigate the expression pattern of TIFA in human colorectal cancer, we performed IHC analyses with a TIFA-specific antibody in human colorectal cancer tissue microarrays containing 180 samples (90 normal colon tissues, 90 colorectal cancer tissues). The results showed that expression levels of TIFA increase in tumor biopsies versus comparable normal tissues (Fig. 1A & 1B). We also explored the correlation between the expression levels of TIFA and the TNM stages of the tumor biopsies. We found a

positive correlation between elevated expression levels of TIFA and high TNM stages tumor biopsies (Fig. 1C). To further confirm this, we also checked the TIFA expression in 12 pairs of fresh colorectal cancer and the adjacent non-tumor tissues by qRT-PCR (Fig. 1D) and Western blot (Fig. 1E), the results showed that TIFA was overexpressed in colorectal cancer tissues and rarely in the adjacent non-tumor tissues. To validate our results, we also analyzed the expression level of TIFA in patients with colorectal cancer in GEPIA and TNMplot online database. We also found that TIFA was overexpressed in colorectal cancer tissues versus comparable normal tissues in both two online databases ($P < 0.05$, Fig. 1F & 1G). Together, the above-mentioned findings suggest that TIFA acts as a potential cancer related mediator, was up-regulated in human colorectal cancer tissues.

Silencing of TIFA inhibits CRC cell proliferation *in vitro*

We investigated whether the observations in clinical samples were represented in tumor cell lines. The mRNA level of TIFA was found to be up-regulated in CRC cell lines relative to normal colon cell line. Immunoblot analysis of TIFA protein levels supported the quantitative PCR results, suggesting that TIFA was increased in CRC cell lines especially in RKO and SW620 cells (Fig. 2A).

To investigate the functional role of TIFA in tumor progression, we knocked down TIFA expression in RKO and SW620 cell lines, as shown in Fig. 2B, the protein level was decreased by using TIFA specific shRNAs. We performed the CCK-8 assay to detect the cell viability and the results showed that knockdown of TIFA expression inhibited the speed of cell growth in the indicated time points (Fig. 2C). We also performed the cell clone formation assay to test the cell proliferation ability (Fig. 2D). We found that knockdown of TIFA expression decreased the cell clone formation ability (Fig. 2E). Moreover, the PI single staining assay was used to analyze the cell cycle progression, the results revealed that TIFA knockdown reduced the amounts of cells in S phase, but increased the amounts of cells in G2/M phase, which further suppressed the cell proliferation ability (Fig. 2F and 2G). In addition, PI-Annexin V double-staining assay was performed to detect the function of TIFA interference in the cell apoptosis. As shown in Fig. 2H, in RKO and SW620 cell lines, knockdown of TIFA expression made no difference on cell apoptosis ability of both cell lines (Fig. 2I). Thus, these results indicated that silencing TIFA inhibits CRC cell proliferation but have no effects on cell apoptosis *in vitro*.

TIFA deficiency suppresses CRC cell proliferation *in vivo*

Since TIFA contributes to the CRC tumor progression via promoting cell proliferation *in vitro*, we next tested the results in the xenograft animal model. To this end, stable RKO-shCtrl, RKO-sh1 and RKO-sh2 cells were injected into the forth fat pad of nude mice, respectively. As shown in Fig. 3A & 3B, the tumor growth and tumor volume in the RKO-sh1 and RKO-sh2 group showed a marked decrease versus the RKO-shCtrl control group. Moreover, consistent with the *in vitro* findings, the IHC staining results also revealed that knockdown TIFA expression reduced the expression of Ki-67 while the expression of apoptosis marker cleaved caspase 3 had no change (Fig. 3C & 3D). These findings collectively suggested that TIFA deficiency suppresses CRC tumor progression *in vivo*.

TIFA mediates RSK and PRAS40 signaling activation *in vitro*

To investigate the underlying mechanism of TIFA mediated CRC cell proliferation, we used a human phosphokinase array that contains 43 kinase phosphorylation sites and 2 related total proteins in RKO-shTIFA and RKO-shCtrl cells. Our results show that TIFA knockdown reduced the expression of many phosphokinases especially p-RSK and p-PRAS40 (Fig. 4A & 4B). Based on this finding, we focused our attention on the p-RSK and p-PRAS40 using a variety of methods. By using western blot, we observed that TIFA knockdown consistently decreased p-RSK and p-PRAS40 expression levels in RKO and SW620 cells (Fig. 4C). Furthermore, using tissue micro-array and immunohistochemistry, we checked the co-expression level of TIFA and p-RSK/p-PRAS40 in tumor tissue micro-array. The results showed that TIFA has a good co-expression with p-RSK and p-PRAS40 (Fig. 4D-4G). In addition, we selected TIFA high expression tumor tissue pairs and checked the expression of p-RSK/p-PRAS40. Consistently, we found that p-RSK and p-PRAS40 also have higher expression in tumor tissues versus the normal control (Fig. 4H). Collectively, these results suggested that TIFA mediates RSK and PRAS40 signaling activation *in vitro* which may further to regulate CRC cell proliferation.

TIFA reconstitution facilitates CRC cell proliferation rely on its oligomerization site and TRAF6 binding site *in vitro*

To further identify the function of TIFA, we reconstituted either wild type TIFA (TIFA), an oligomerization site mutant TIFA (T9A) or TRAF6 binding site deletion TIFA (D6) (Fig. 5A) into SW480 cell lines. Western results showed that the three types of TIFA constructs were all reconstituted in SW480 cells (Fig. 5B). CCK8 results noticed that the cell proliferation ability was increased upon the expression of TIFA and this effect was abolished by the expression of TIFA-T9A and TIFA-D6 (Fig. 5C). Moreover, cell clone formation results displayed that TIFA reconstitution accelerated the cell growth at the indicated time points; however, the TIFA-T9A and TIFA-D6 showed no effects (Fig. 5D & 5E). In addition, the PI single staining results revealed that TIFA reconstitution increased the amounts of cells in S phase, reduced the amounts of cells in G2/M phase, while TIFA-T9A and TIFA-D6 abolished this effect (Fig. 5F & 5G). In agreement, the western results showed that the expression of proliferation specific protein Ki67, p-RSK and p-PRAS40 were increased in TIFA reconstitution group but have no change in TIFA-T9A and TIFA-D6 groups (Fig. 5B). In summary, these results disclosed that TIFA reconstitution fosters CRC cell proliferation and downstream signaling activation depend on its oligomerization site and TRAF6 binding site *in vitro*.

TIFA mediates p-RSK/p-PRAS40 activation to foster CRC tumor progression rely on its oligomerization site and TRAF6 binding site *in vivo*

Since TIFA mediates p-RSK/p-PRAS40 activation to contribute to CRC cell proliferation *in vitro*, we next tested the results in a xenograft animal model. To this end, stable SW480-OEctrl, SW480-TIFA, SW480-TIFAT9A and SW480-TIFAD6 cells were injected into the forth fat pad of nude mice, respectively. As shown in (Fig. 6A & 6B) the tumor growth and tumor volume in the SW480-TIFA group showed a marked increase versus the SW480-OEctrl control; however, a significant reduction of the tumor growth and tumor volume was observed in SW480-TIFAT9A and SW480-TIFAD6 groups versus the SW480-TIFA group.

Moreover, the IHC staining results also revealed that TIFA reconstitution increased the expression of Ki-67, p-RSK and p-PRAS40 while these effects were abolished in SW480-TIFAT9A and SW480-TIFAD6 groups (Fig. 6C & 6D). These findings collectively suggested that TIFA mediates p-RSK/p-PRAS40 activation to foster CRC tumor progression rely on its oligomerization site and TRAF6 binding site *in vivo*.

RSK and PRAS40 activation were indispensable for TIFA mediated CRC cell proliferation in vitro

To investigate this role of RSK and PRAS40 activation in TIFA mediated CRC cell proliferation, we used the specific inhibitor for RSK and specific shRNAs for PRAS40 to suppress their phosphorylation and activation. As shown in Fig. 7A, the inhibitor BRD7389 (4uM) inhibits RSK activation effectively in SW480-TIFA cells. As shown in Fig. 7B, the PRAS40-shRNAs inhibits PRAS40 expression and activation efficiently in SW480-TIFA cells as well. Further we combined the BRD7389 (4uM) and PRAS40-shRNA to treat the SW480-TIFA cells, then checked the expression of Ki-67, p-RSK and p-PRAS40 correspondingly. The western blot results showed that BRD7389 (4uM), PRAS40-shRNA or combining treatment reduced the expression of Ki-67 apparently (Fig. 7C). Moreover, the CCK8 assay results displayed that the cell proliferation ability enhanced upon TIFA expression was abolished by the BRD7389 (4uM), PRAS40-shRNA or combining treatment (Fig. 7D). Furthermore, the cell clone formation assay results showed that the cell growth ability reinforced upon TIFA expression was also reduced by the BRD7389 (4uM), PRAS40-shRNA or combining treatment (Fig. 7E & 7F). The same results were proved by the the PI single staining assay, the results revealed that the cell cycle acceleration mediated by TIFA was decelerated by the BRD7389 (4uM), PRAS40-shRNA or combining treatment as well (Fig. 7G & 7H).

To investigate the potential mechanism of TIFA mediated RSK and PRAS40 activation, the immunoprecipitation assay was performed to detect the co-expression of Flag-TIFA, TRAF6, RSK and PRAS40. The results claimed that TIFA could bind TRAF6 and RSK to mediate RSK activation, but PRAS40 was not examined (Fig. 7I). Altogether, these findings suggested that RSK and PRAS40 activation were indispensable for TIFA mediated CRC cell proliferation. RSK and PRAS40 worked as the potential therapy targets for TIFA mediated CRC progression.

Proposed model of TIFA promotes CRC cell proliferation

Based on the totality of our findings, we propose the following model (Fig. 7J): TIFA acts as an adaptor protein, its oligomerization mediate the oligomerization of binding protein TRAF6, further to activate RSK kinase directly and PRAS40 indirectly, the activated RSK and PRAS40 could facilitate protein synthesis in cell cycle, finally to promote cell proliferation and foster CRC tumor progression.

Discussion

As one of the major common human malignancies, Colorectal cancer (CRC) has been ranked as the third most common diagnosed cancer in males and the second in females according to morbidity and mortality, respectively [1]. Moreover, clinical success in the pharmacological treatment of CRC patients

has been limited. With the precision medicine developed, it is in urgent need to explore novel new prognosis biomarkers or therapeutic targets for CRC clinical treatment.

TIFA acts as an inflammation related adaptor protein, plays a vital role in various biological processes which contain signal transduction in innate immune [9, 36–39], inflammation[40, 41], hypoxia-reoxygenation [42] and cancer. Previous studies proved that TIFA plays different roles in various cancer types. As our previous work described, TIFA expression is down-regulated in HCC and TIFA reconstitution promotes HCC cell apoptosis, suppresses cell proliferation among surviving cells [12, 13]. However, other studies revealed that TIFA is high-expressed in lung adenocarcinoma, which could promote lung adenocarcinoma cell survival and migration [11]. Moreover, TIFA is proved to be over-expressed in acute myeloid leukemia (AML), correlated with poor prognosis and also contributed to chemo-resistance [43]. However, the function of TIFA in colorectal cancer (CRC) was not dissected. In this study, we showed that the expression of TIFA was marked up-regulated in CRC tissues and cell lines versus normal tissue or normal cell line. TIFA expression was also positively correlated with CRC TNM stages. That's may suggest that TIFA acts as a promising biomarker for CRC prognosis.

Cell cycling and protein synthesis are both key physiological processes for tumor cells proliferation [14]. Protein synthesis is occurred in G0/G1 (enzymes like CDKs) and G2/M phase (cyclins and others) of cell cycle. RSK comprises a family of serine/threonine protein kinases was proved to regulate the stability of eIF to mediate protein synthesis [20]. PRAS40 identified as a component and substrate of the mTOR complex 1 was also showed to regulate protein synthesis process [24]. Although some previous studies have proved that RSK and PRAS40 activation was involved in colon inflammation and CRC progression, in this study, it should be highlighted that TIFA mediates RSK and PRAS40 activation not only reveal novel new mechanism for TIFA function but also provide therapeutic strategy for CRC. In the exploration of the underlying mechanism, we found that the oligomerization site (T9) and TRAF6 binding site (E178) were all indispensable for RSK and PRAS40 activation. Moreover, we showed that TIFA-TRAF6 complex binds and activates RSK directly. While PRAS40 activation mediated by TIFA-TRAF6 complex was indirectly which need to be investigated in the future study. In addition, we used the RSK inhibitor and PRAS40 shRNAs to prevent RSK and PRAS40 activation and found that they suppressed TIFA mediated CRC cell proliferation significantly. That's may provide the potential therapy targets and strategy for CRC progression.

Conclusions

In conclusion, we discovered that TIFA promotes cell proliferation in CRC cell line *in vitro* and in a xenograft nude mouse model *in vivo*. We revealed that TIFA mediates RSK and PRAS40 activation to facilitate protein synthesis which relied on TIFA oligomerization (T9) and TRAF6 binding (E178) *in vitro* and *in vivo*. We also demonstrated that RSK inhibitor and PRAS40 shRNAs which prevent RSK and PRAS40 activation could suppress TIFA mediated CRC cell proliferation significantly. Finally, we take the position that TIFA may serve as a novel therapeutic target in CRC treatment.

Abbreviations

TIFA
TNF receptor associated factor (TRAF)-interacting protein with a Forkhead-associated (FHA) domain
CRC
Colorectal cancer
CCK-8
Cell counting kit-8
RSK
Ribosomal S6 protein kinase
PRAS40
Proline-Rich Akt Substrate of 40 kDa
TRAF6
TNF receptor associated factor 6.

Declarations

Ethics approval and consent to participate

The CRC tissues we used were obtained from the patients who underwent surgical treatment at the Affiliated Hospital of Jining Medical University from 2015 to 2019. All tissues from the patients were staged based on the American Joint Committee on Cancer TNM staging system. This research was approved by the institutional ethics committees at the Jining Medical University.

Consent for publication

Not applicable.

Competing interest

The authors declare no potential conflicts of interest.

Author Contributions

SWZ and DWF designed the experiments, LYP and HYM did bio-informatics, JXY, YCL, TJP, YR and WSS helped to analyze the data, LH, YSY and LN provided methods or small inhibitors, SWZ, ZXY and ZZX prepared and repaired the manuscripts.

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Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author in response to reasonable requests.

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Figures

Figure 1

Figure 1

TIFA was up-regulated in human colorectal cancer tissues. A. Representative immunohistochemistry of TIFA in a human colorectal cancer tissue array. Scale bar: 50 μ m B. Quantification of TIFA immunohistochemistry in a CRC tissue array. C. Quantification of TIFA immunostaining and TNMs. D & E. Representative qRT-PCR and Western blot analysis of 12 pairs of fresh colon cancer tissue and normal

adjacent tissue. F & G. Transcriptional expressions of TIFA in CRC cancers (cancer vs. corresponding normal tissue) were evaluated using the GEPIA and TNMplot online database.

Figure 2

Figure 2

Silencing of TIFA inhibits CRC cell proliferation in vitro. A. QPCR and Western blot analysis of TIFA expression in CRC cell lines and normal colon cell lines. B. Western blot analysis TIFA knockdown in RKO and SW620 cell lines and the expression of Ki-67, cleaved caspase 3, β -actin was included as a loading

control. C. CCK8 assay was performed to detect cell viability in RKO and SW620 cells with silencing TIFA or shCtrl. D. Cell clone formation assay to test the cell proliferation ability in RKO and SW620 cells with silencing TIFA or shCtrl. E. The statistical results of cell clone formation assay were shown. F. PI single staining assay was used to analyze the cell cycle progression in RKO and SW620 cells with silencing TIFA or shCtrl. G. The statistical results of PI single staining assay were shown. H. PI-Annexin V assay was performed on shCtrl and shTIFA of RKO and SW620 cells. A representative of three experiments was shown. I. The statistical results of PI-Annexin V assay were shown.

Figure 3

Figure 3

TIFA deficiency suppresses CRC cell proliferation in vivo. A. stable RKO-shCtrl, RKO-shTIFA-1 and RKO-shTIFA-2 cells were injected into the forth fat pad of nude mice, the tumor growth curve was measured. B. The tumors from different group mice were shown. C. IHC staining assay was performed to detect the

expression of TIFA, Ki-67 and cleaved caspase 3 in various group mice tumors. D. The statistical results of the TIFA, Ki-67 and cleaved caspase 3 positive cells in various group mice tumors were shown.

Figure 4

Figure 4

TIFA mediates RSK and PRAS40 signaling activation in vitro. A. Human phosphokinase array was performed to detect 43 kinases activation in RKO cells with silencing TIFA or shCtrl. B. 7 kinases or proteins were selected and the statistical results were shown. C. Western blot analysis the expression of

RSK, p-RSK, PRAS40 and p-PRAS40, β -actin was included as a loading control. D & F. Representative immunohistochemistry of TIFA, p-RSK and correlation graphs in a human colorectal cancer tissue array were shown. Scale bar: 50 μ m E & G. Representative immunohistochemistry of TIFA, p-PRAS40 and correlation graphs in a human colorectal cancer tissue array were shown. Scale bar: 50 μ m H. Western blot analysis the expression of p-RSK and p-PRAS40 in TIFA high-expressed fresh colon cancer tissue, β -actin was included as a loading control.

Figure 5

Figure 5

TIFA reconstitution facilitates CRC cell proliferation rely on its oligomerization site (T9) and TRAF6 binding site (E178) in vitro. A. Schematic structure of oligomerization site mutant TIFA (T9A) and TRAF6 binding site mutant TIFA (D6) was shown. B. Western blot analysis TIFA overexpression in SW480 cell line and the expression of Ki-67, RSK, p-RSK, PRAS40 and p-PRAS40, β -actin was included as a loading control. C. CCK8 assay was performed to detect cell viability in SW480 cells with reconstitute TIFA or OECtrl. D. Cell clone formation assay to test the cell proliferation ability in SW480 cells with reconstitute TIFA or OECtrl. E. The statistical results of cell clone formation assay were shown. F. PI single staining assay was used to analyze the cell cycle progression in SW480 cells with reconstitute TIFA or OECtrl. G. The statistical results of PI single staining assay were shown.

Figure 6

Figure 6

TIFA mediates p-RSK/p-PRAS40 activation to foster CRC tumor progression rely on its oligomerization site and TRAF6 binding site in vivo. A. stable SW480-OECtrl, SW480-OETIFA, SW480-OETIFA T9A and SW480-OETIFA D6 cells were injected into the forth fat pad of nude mice, the tumor growth curve was measured. B. The tumors from different group mice were shown. C. IHC staining assay was performed to detect the expression of FLAG, TIFA, Ki-67 p-RSK and p-PRAS40 in various group mice tumors. D. The

statistical results of the FLAG, TIFA, Ki-67 p-RSK and p-PRAS40 positive cells in various group mice tumors were shown.

Figure 7

Figure 7

RSK and PRAS40 activation were indispensable for TIFA mediated CRC cell proliferation in vitro. A. Western blot was performed to check the inhibition of BRD7389 to RSK activation in SW480-OETIFA cells, the concentration was 0uM, 4uM, 8uM respectively. B. Western blot analysis the inhibition of PRAS40-

shRNAs to PRAS40 activation in SW480-OETIFA cells. C. Western blot analysis the expression of RSK, p-RSK, PRAS40, p-PRAS40 and Ki-67 in SW480-OETIFA treated with BRD7389 or PRAS40-shRNA or BRD7389 combine PRAS40-shRNA cells. D. Cell clone formation assay to test the cell proliferation ability in SW480-OETIFA treated with BRD7389 or PRAS40-shRNA or BRD7389 combine PRAS40-shRNA cells. E. Cell clone formation assay to test the cell proliferation ability in SW480-OETIFA treated with BRD7389 or PRAS40-shRNA or BRD7389 combine PRAS40-shRNA cells. F. The statistical results of cell clone formation assay were shown. G. PI single staining assay was used to analyze the cell cycle progression in SW480-OETIFA treated with BRD7389 or PRAS40-shRNA or BRD7389 combine PRAS40-shRNA cells. H. The statistical results of PI single staining assay were shown. I. Immunoprecipitation assay was performed to detect the co-expression of Flag-TIFA, TRAF6, RSK and PRAS40. J. Proposed model of TIFA promotes CRC cell proliferation.

Supplementary Files

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