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Original Article

Knockdown of YEATS4 inhibits colorectal cancer cell proliferation and induces apoptosis

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Abstract: YEATS domain containing 4 (YEATS4) is usually amplified and functions as an oncogene in human glioma. However, the biological role of YEATS4 in colorectal cancer (CRC) has not yet been discussed. In this study, we investigated the expression level of YEATS4 in 85 pairs of CRC and paracancerous tissues, and knocked down YEATS4 via a lentivirus system in RKO CRC cell line. Although YEATS4 was upregulated in CRC tissues, YEATS4 expression showed no association with any clinical features and overall survival. Inhibition of YEATS4 significantly suppressed cell proliferation and colony formation. Flow cytometry revealed that cell cycle was arrested in the G0/G1 phase and the number of apoptotic cells were significantly increased when YEATS4 expression was inhibited. In conclusion, our findings provide first evidence that YEATS4 may be an important regulator of cell proliferation and apoptosis in CRC cells.

Keywords: YEATS domain containing 4, colorectal cancer, apoptosis, proliferation, cell cycle

Introduction

Colorectal cancer (CRC) is one of the most common forms of cancer worldwide, accounting for 9.4% of the total cancer cases and 8.0% of the cancer deaths [1]. Tumorigenesis is a complex biological process that is closely related to the activation of oncogenes and inactivation of tumor suppressor genes. Clarification of the underlying molecular mechanism of tumor occurrence and identification of effective targets can help in the diagnosis and treatment of CRC.

YEATS domain containing 4 (YEATS4) is a highly conserved nuclear protein that is originally identified in human glioma [2]. By investigating the proteins that interact with YEATS4, it can be inferred that YEATS4 is involved in chromatin modification and transcriptional regulation [3, 4]. Many YEATS4 binding partners have been identified, including AF10, KIAA1009, MYC, MYCN, NuMa, TACC1, TACC2, AP-2beta, TFIIF, and PFDN1 [5-9]. YEATS4 functions as an oncogene and is amplified in some types of malignancy such as non-small cell lung cancer [10], astrocytomas [2, 11], glioblastomas [11], and liposarcoma [12].

Only a few reports about YEATS4 are available, and the function of YEATS4 in CRC has not been reported yet. In this study, we investigated YEATS4 expression in CRC tissues and examined the effect of YEATS4 on CRC cells proliferation, cell cycle, apoptosis and migration. The biological significance of YEATS4 in CRC was investigated to provide experimental evidence for targeted therapy of CRC.

Materials and methods

Patients and tissue samples

A total of 85 pairs of CRC and paracancerous tissues were derived from patients undergoing CRC resection surgeries at Tongren Hospital between 2008 and 2014. All samples were confirmed by two independent pathologists. Written informed consent was obtained from all patients, and the protocol was approved by the Ethics Committees of Tongren Hospital.

Cell lines

Human CRC cell lines RKO, HCT116, HT29, and DLD-1 were obtained from the American Type Culture Collection (ATCC). Cell lines were main-

tained in DMEM (Gibco, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin at 37°C in a humidified incubator with 5% CO_2.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from CRC tissues and cell lines using Trizol reagent (Invitrogen, CA, USA) according to manufacturer's instruction. The mRNA level of YEATS4 was determined by a qRT-PCR assay. The following primers were used for qRT-PCR: YEATS4, 5'-TCATAGAACTCTGAAACCACTGTC-3' (sense) and 5'-TCATAGAACTCTGAAACCACTGTC-3' (antisense); GAPDH, 5'-TGACTTCAACAGCGACACCCA-3' (sense), 5'-CACCCTGTTGCTGTAGCCAAA-3' (antisense). qRT-PCR was carried out using SYBR Premix Ex TaqII kit (Takara, Dalian, China) in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, CA, USA). All RT-PCRs were performed in triplicates. The relative expression level of YEATS4 was normalized to GAPDH and calculated using the formula $2^{-\Delta\Delta Ct}$.

Western blot

Cells were lysed in an ice-cold lysis buffer containing 50 mM Tris, 2% 2-mercaptoethanol, 20% glycerol, and 4% SDS. Lysates were centrifuged at 12,000 × *g* at 4°C for 15 min, and the protein concentrations in the supernatants were determined using by Bio-Rad protein assays. After denaturation by heating, the proteins were separated by electrophoresis with 10% SDS-PAGE and then transferred onto PVDF membranes. Blots were blocked, and rabbit anti-human antibodies was added. Membranes were incubated at 4°C overnight and washed, and then peroxidase-conjugated secondary antibodies were added. Finally, Membranes were incubated at room temperature for 2 h. Blots were visualized using ECL Plus Western blotting detection kit (Amersham Pharmacia Biotech, NJ, USA). GAPDH was used as a loading control.

RNA interference

YEATS siRNA target sequences was 5'-GCTGTTTCAATCAGACACCAA-3'. The siRNA was synthesized by Shanghai Genechem Co., Ltd., China. RKO cells growing in good condition were cultured, and 5×10^4 cells were inoculated into each well of six-well plates a day before virus infection. The cells were cultured in an incubator at 37°C with 5% CO_2. On the day of infection, the experiment on RKO cells was conducted by adding RNAi lentiviral particles. GFP expression was observed with a fluorescence microscope 3 d after infection, and the cells were collected 5 d after infection. The interference effects of the target were assessed using qRT-PCR and Western blot.

Cell proliferation assay

Cells in logarithmic growth phase were digested into a single-cell suspension, and 3000 cells were inoculated into each well of 96-well plates. The plates were cultured in an incubator at 37°C with 5% CO_2. From the next day after plating, the plates were detected and read once per day for five consecutive days. The number of green fluorescent cells in the plates at each scan was calculated accurately to draw the cell proliferation curve within 5 d.

MTT assay

Cells in logarithmic growth phase were digested into a single-cell suspension, and 3000 cells were inoculated into each well of 96-well plates, with six wells in each group. The plates were cultured in an incubator at 37°C with 5% CO_2. The absorbance was determined by MTT at days 1, 2, 3, 4, and 5. Exactly 100 μL of 0.2 mg/mL MTT solution (Dingguo, Beijing, China) was added into each well for 3 h of continuous culture, and the supernatant was discarded. Finally, 150 μL of DMSO (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was added into each well, and the mixture was shocked fully for 10 min. Colorimetric assay with OD value at 490/570 nm was conducted with a microplate reader Elx800 (Biotek, VT, USA) to draw the growth curve.

Colony formation assay

Cells in logarithmic growth phase were digested into a single-cell suspension, and 800 cells were inoculated into each well. Transfection was performed using the same multiplicity of infection (MOI) after 24 h. Subsequently, the cells were cultured statically at 37°C with 5% CO_2 for two weeks, fixed in methanol, and stained with Giemsa. The clones were counted, and the colony forming efficiency was calculated by the formula: (colony forming efficiency = number of clone/inoculated cell number × 100%).

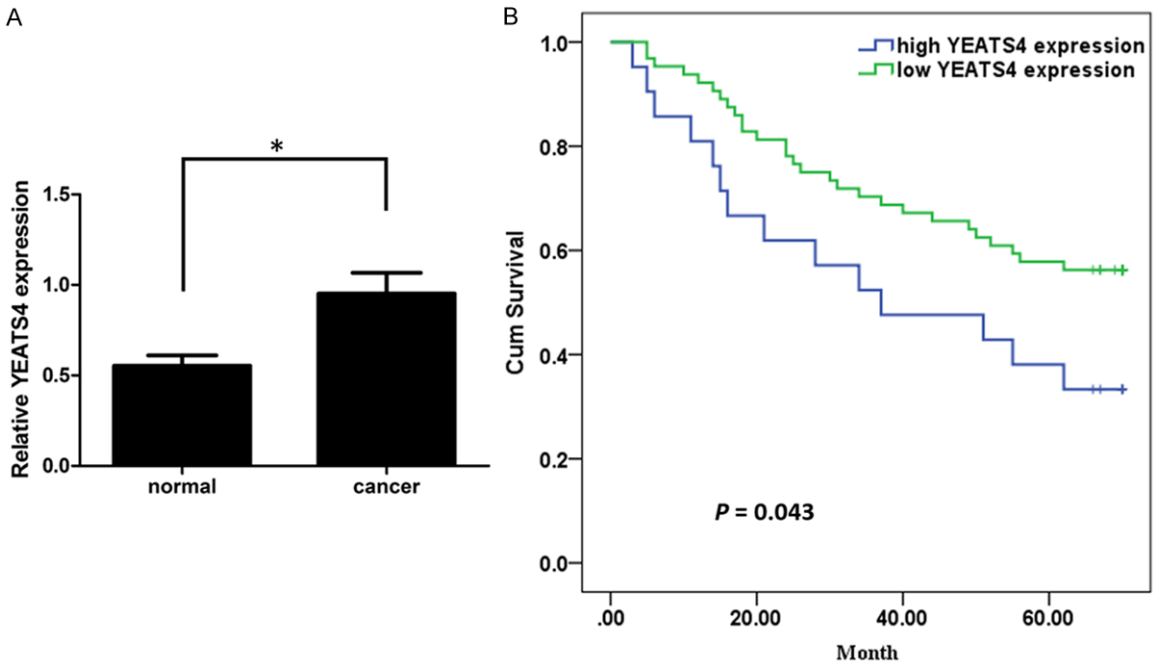

Figure 1. YEATS4 expression in CRC tissues and its clinical significance. A. YEATS4 was significantly upregulated in CRC tissues compared with adjacent normal tissues. B. Kaplan-Meier overall survival curves according to YEATS4 expression level. The median survival time of patients with high YEATS4 expression (n = 21: YEATS4 expression > 4th quartile) was significantly lower than that of patients with low YEATS4 expression (n = 64: MEG3 expression ≤ 4th quartile). *P* = 0.043, log-rank test. **P* <0.05.

Flow cytometry/cell cycle analysis

When cell fusion reached 80%, the cells were digested into a single-cell suspension. Apoptosis was detected using Annexin V-APC apoptosis detection kit (eBioscience, CA, USA) according to the manufacturer's instructions. The cell cycle was determined using the conventional staining method. Specifically, the cells were fixed in 70% ethanol pre-cooled for 1 h, centrifuged at 1500 g for 5 min to remove the fixation solution, and washed once with PBS. The cells were then collected after centrifugation at 1500 g for 5 min, and RNase A (Sigma, MO, USA) was added to 1 mL of the pre-cooled resuspended cells in PBS to obtain a final concentration of 0.25 g/L. The resulting suspension was placed at 37°C for 30 min, and the cells were stained with 5 mL of PI (Sigma, MO, USA) at room temperature in the dark for 5 min. Finally, the cell cycle was detected at 488 nm with FACSCalibur flow cytometer (BD, NJ, USA).

Transwell migration assay

A 24-well transwell chamber (8 μm) was adopted for the cell migration experiment. The cell concentration was adjusted to 1×10^6/mL with serum-free medium 24 h after cell treatment with serum-free medium. Subsequently, 100 μL of cell suspension in each group was added into the chambers, and 600 μL of 30% fetal bovine serum was added into the lower chamber. After 24 h of incubation, the non-metastatic cells on the upper chamber were wiped and then stained with crystal violet. The average transmembrane cell number of five fields was counted. This experiment was repeated three times.

Statistical analyses

All statistical analyses were performed using SPSS software, version 19.0 (SPSS Inc, IL, USA). Quantitative data were presented as the mean ± SD, and tested for significance with the Student t test. The expression levels of YEATS4 in CRC and paracancerous tissues were compared using the Wilcoxon signed-rank test. The relationship between YEATS4 expression and clinical features was tested using Chi-square test or Fisher's exact test. The impact of YEATS4 expression on overall survival was evaluated using Kaplan–Meier and log-rank analysis. Cox's proportional hazard model was per-

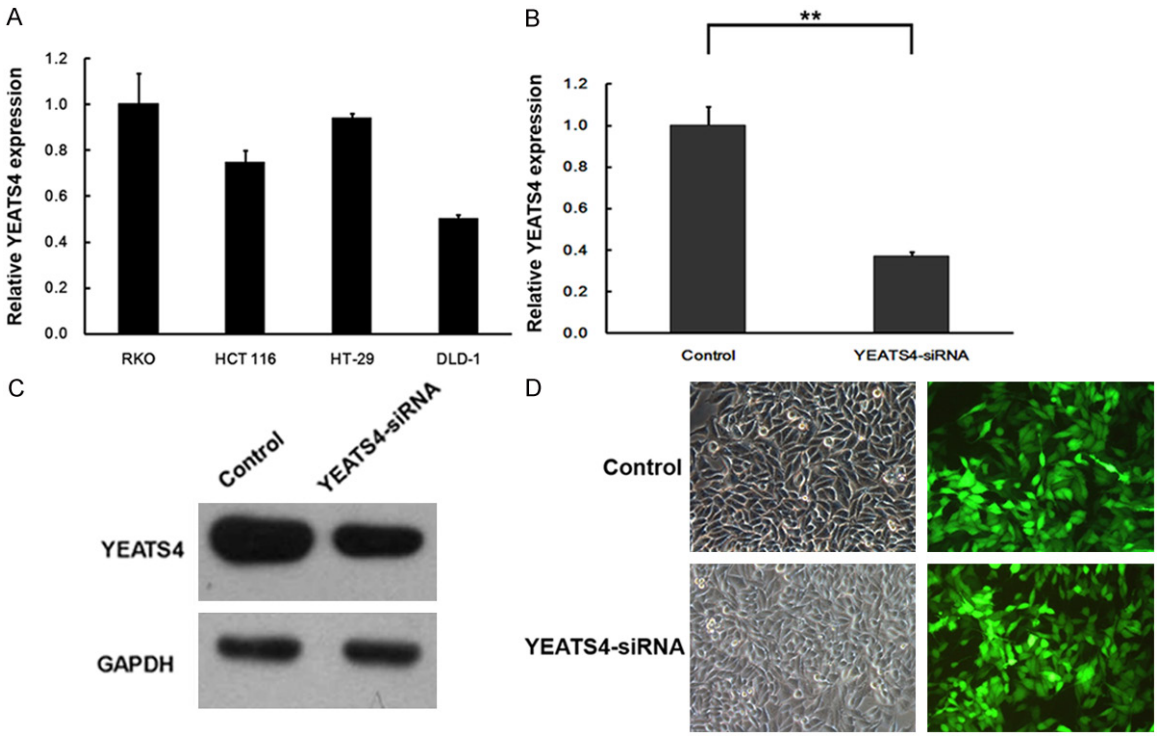

Figure 2. Knockdown of YEATS4 expression in RKO cells. A. The mRNA level of YEATS4 was detected in CRC tissues and cell lines by qRT-PCR. B & C. knockdown of YEAST4 in RKO cells was confirmed by qPCR and Western blotting, respectively. D. The expression of green fluorescent protein in transfected cells was detected by fluorescence microscopy (× 200). $^{**}P < 0.01$.

formed to calculate hazard ratio (HR) and 95% confidence interval (CI). All tests were two-tailed and a *P* values of < 0.05 was considered statistically significant.

Results

Dissecting YEATS4 expression in clinical samples

Although YEATS4 has been reported as overexpressed in some human cancers such as lung adenocarcinoma [10], its expression has not been extensively investigated in CRC. The mRNA levels of YEATS4 in CRC tissues were therefore compared against adjacent normal tissues. The levels of YEATS4 in CRC tissues were significantly higher than those in paracancerous tissues ($P = 0.027$, **Figure 1A**). However, there was no difference between YEATS4 expression and any clinical features (data not shown). Furthermore, the impact of YEATS4 expression on survival in 86 CRC patients was evaluated. In Kaplan-Meier analysis, there was a weak but significant association between YEATS4 expression and overall survival ($P = 0.043$, **Figure 1B**). Cox proportional hazards regression analysis revealed that high YEATS4 expression (≥ 4th quartile) was significantly correlated with worse survival time (HR = 1.910, 95% CI: 1.005-3.632, $P = 0.048$). However, the difference disappeared after adjustment for age, sex, histological grade, size, and TNM stage.

YEATS4 regulates the proliferation and colony formation of CRC cells

The level of YEATS4 was investigated in CRC cell lines RKO, HCT116, HT-29, and DLD-1, and RKO cells showed the highest level of YEATS4 (**Figure 2**). Consequently, RKO cell line was chose for the follow-up experiments. To explore the function of YEATS4 in the progression of CRC, lentiviral shRNA targeting YEATS4 was stably transfected into RKO cells. The mRNA and protein levels of YEATS4 were confirmed by qPCR and Western blot, respectively. The YEATS4 siRNA effectively down-regulated the expression levels of YEATS4 in RKO cells. YEATS4 silencing significantly reduced cell viability ($P < 0.05$, **Figure 3A**), indicating that

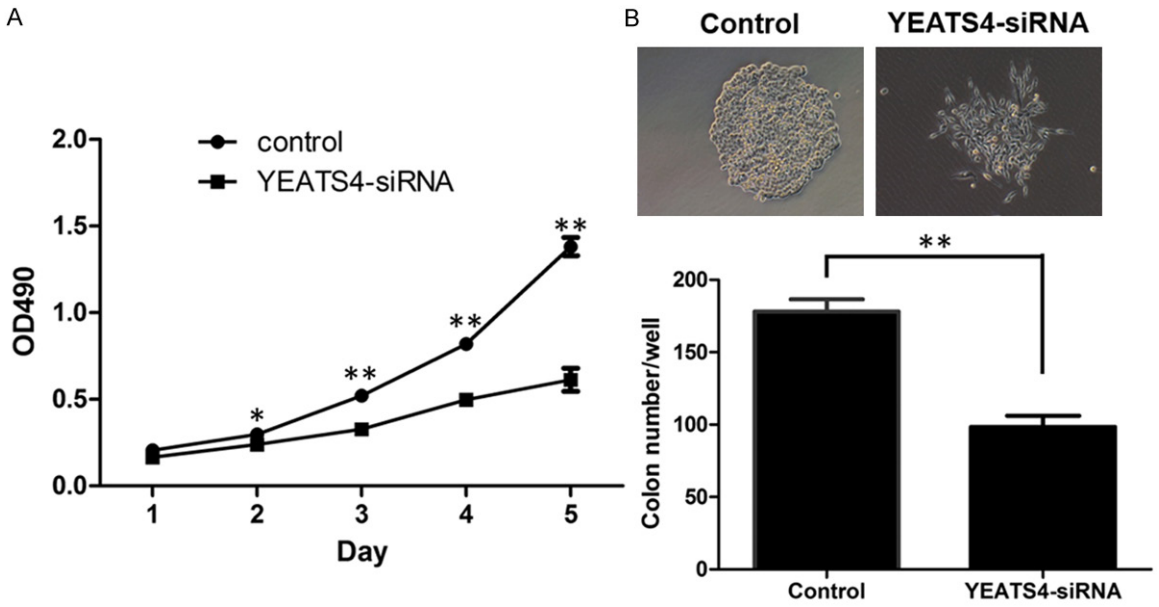

Figure 3. Effects of YEATS4 knockdown on cell proliferation and colony formation in RKO cells. A. Cells proliferation was determined by MTT assay. Inhibition of YEATS4 suppressed growth of RKO cells in a time-dependent manner. B. Colony formation assay was performed to determine the proliferation potential of cells. The number of colonies in YEATS4-scilenced RKO cells was significantly reduced compared with the controls. Data were shown as mean ± SD of triplicate experiments, $*P < 0.05$, $**P < 0.01$.

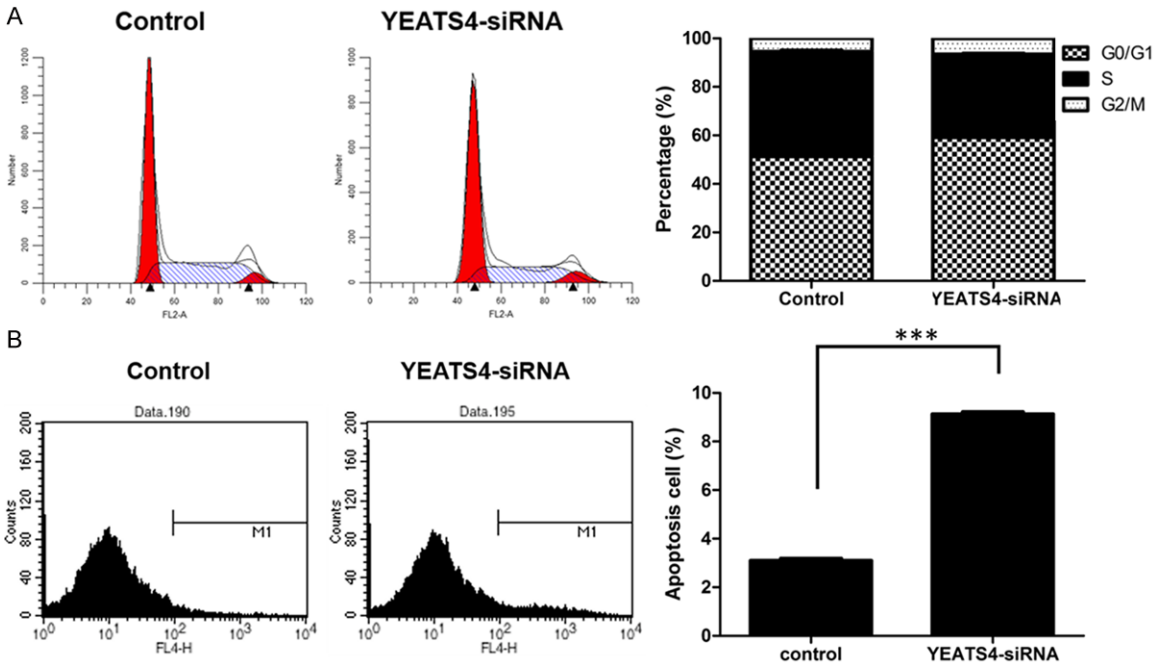

Figure 4. Effects of YEATS4 knockdown on cell cycle and apoptosis of RKO cells. A. The bar chart represented the percentage of cells in G0/G1, S, or G2/M phase, as indicated. B. Inhibition of YEATS4 promoted RKO cells apoptosis. $***P < 0.001$.

YEATS4 may promote CRC cell proliferation. Similar to anti-proliferation by YEATS4 siRNA, in a colony-forming assay, the members of YEATS4-silenced RKO cell colonies were significantly reduced compared with the negative control ($P < 0.01$, **Figure 3B**).

YEATS4 regulates the cell cycle and apoptosis of CRC cells

Cell cycle analysis showed that knockdown of YEATS4 caused increased accumulation of cells in G0/G1 ($P < 0.01$), with reductions in both the S ($P < 0.01$) and G2/M populations (**Figure 4A**). This result indicated that inhibition of YEATS4 in RKO cells affected their normal regulation of cell cycle progression. Cell death analysis revealed an increase in apoptosis rate of RKO cells as visualized through Annexin-V positive cells when YEATS4 expression was inhibited ($P < 0.001$, **Figure 4B**).

The effect of YEATS4 knockdown on the migration of CRC cells

We further performed *in vitro* loss-of-function analyses to determine the role played by YEATS4 in RKO cell migration. Transwell migration assay showed no difference in migration ability between YEATS4 knockdown and negative control groups ($P > 0.05$, data not shown).

Discussion

YEATS4, originally identified in glioblastoma multiform cell line TX3868, is usually amplified in astrocytomas [2, 11] and glioblastomas [11]. Therefore, it is considered to act as an oncogene in the development of glioma. YEATS4 is located in chromosome 12q13-15, which is amplified in CRC [13, 14]. In this study, we analyzed the expression level of YEATS4 in CRC tissues and explored the effects of YEATS4 on CRC cell lines. YEATS4 is upregulated in CRC and promotes CRC cells proliferation. However, the expression level of YEATS4 did not affect prognosis of CRC.

YEATS4 contains 227 amino acids and has a molecular weight of 26.7 KD. YEATS4 has a negatively charged α-helical structure at the C-terminal end, which is the typical feature of eukaryotic transcription factor activation domain and is also the protein-protein interaction area [2]. The N-terminal domain consists of the tf2f domain, which is highly conserved in the YEATS protein family [13]. Given that YEATS4 lacks the DNA binding domain and only contains the DNA activation domain, it is predicted to be an incomplete transcription factor. For example, Ding X. et al. [9] reported that YEATS4 can function as a transcriptional co-activator for AP-2β to enhance its DNA-binding activity. YEATS4 can interact with MLL infusion protein AF10 via the C-terminal activation domain, and is involved in the development and progression of leukemia [6]. YEATS4 overexpression leads to inhibition of senescence and an increase in multipolar mitotic spindle formation [10, 14]. Furthermore, Zimmermann et al. [15] reported that YEATS4 knock out caused chicken pre-lymphoid cell line DT40 cells death, indicating that YEATS4 is required for RNA transcription and is essential for cell viability. More recent study has revealed that YEATS4 is a critical RNAi component [16]. Given that its important role in the processes of transcription regulation, YEATS4 is involved in many cellular procession such as cell proliferation, differentiation and carcinogenesis.

To explore the effect of the YEATS4 gene on CRC, we adopted RNAi technology to silence YEATS4 expression in RKO cells. We found that inhibition of YEATS4 reduced the rate of cell proliferation through cell cycle arrest during the G1 phase and induced apoptosis in RKO cells. Therefore, the YEATS4 gene could promote CRC cell proliferation by inhibiting apoptosis pathway, which is an important factor that promotes the occurrence and development of colon cancer. Llanos et al. reported that YEATS4 was involved in the regulation of p53 activity, and can inhibit p53-mediated apoptosis [17]. A study by Pikor et al. [10] showed that YEATS4 acted as a negative regulator of the p21-p53 pathway to promote lung cancer cells proliferation and inhibit apoptosis. Nevertheless, YEATS4 may regulate P53 activity through multiple approaches. Under non-stress and normal conditions, YEATS4 binds to the promoter of p14 and p21 to inhibit their expression, thereby repressing the p53 pathway [18]. This mechanism does not rely on the action of TIP60 HAT complex and co-activator. As a consequence, YEATS4 is speculated to block the abnormal activation of growth suppressor, such as p14 and p21, and it may be involved in the inhibition of the p53 tumor suppressor pathway in normal cell cycle. Furthermore, YEATS4 can bind to PP2Cβ, a PP2C family member, to form a complex, which dephosphorylates serine 366 on p53 and regulates its stability [19]. Further studies are required to elucidate mechanism by which YEATS4 regulates cell proliferation and apoptosis of CRC.

In conclusion, to the best of our knowledge, this is the first report describing the role of YEATS4 in CRC. Our findings provide preliminary evidence that inhibition of YEATS4 by RNA interference suppresses the proliferation and effectively induces the apoptosis in CRC cells. This offers experimental evidence to gene therapy of CRC. However, further studies are needed to explore whether YEATS4 is an effective target for the clinical treatment of CRC.

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Disclosure of conflict of interest

None.

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References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Fischer U, Heckel D, Michel A, Janka M, Hulsebos T and Meese E. Cloning of a novel transcription factor-like gene amplified in human glioma including astrocytoma grade I. Hum Mol Genet 1997; 6: 1817-1822.

[3] Cai Y, Jin J, Florens L, Swanson SK, Kusch T, Li B, Workman JL, Washburn MP, Conaway RC and Conaway JW. The mammalian YL1 protein is a shared subunit of the TRRAP/TIP60 histone acetyltransferase and SRCAP complexes. J Biol Chem 2005; 280: 13665-13670.

[4] Doyon Y, Selleck W, Lane WS, Tan S and Cote J. Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. Mol Cell Biol 2004; 24: 1884-1896.

[5] Heisel S, Habel NC, Schuetz N, Ruggieri A and Meese E. The YEATS family member GAS41 interacts with the general transcription factor TFIIF. BMC Mol Biol 2010; 11: 53.

[6] Debernardi S, Bassini A, Jones LK, Chaplin T, Linder B, de Bruijn DR, Meese E and Young BD. The MLL fusion partner AF10 binds GAS41, a protein that interacts with the human SWI/SNF complex. Blood 2002; 99: 275-281.

[7] Harborth J, Weber K and Osborn M. GAS41, a highly conserved protein in eukaryotic nuclei, binds to NuMA. J Biol Chem 2000; 275: 31979-31985.

[8] Lauffart B, Howell SJ, Tasch JE, Cowell JK and Still IH. Interaction of the transforming acidic coiled-coil 1 (TACC1) protein with ch-TOG and GAS41/NuBI1 suggests multiple TACC1-containing protein complexes in human cells. Biochem J 2002; 363: 195-200.

[9] Ding X, Fan C, Zhou J, Zhong Y, Liu R, Ren K, Hu X, Luo C, Xiao S, Wang Y, Feng D and Zhang J. GAS41 interacts with transcription factor AP-2beta and stimulates AP-2beta-mediated transactivation. Nucleic Acids Res 2006; 34: 2570-2578.

[10] Pikor LA, Lockwood WW, Thu KL, Vucic EA, Chari R, Gazdar AF, Lam S and Lam WL. YEATS4 is a novel oncogene amplified in non-small cell lung cancer that regulates the p53 pathway. Cancer Res 2013; 73: 7301-7312.

[11] Fischer U, Meltzer P and Meese E. Twelve amplified and expressed genes localized in a single domain in glioma. Hum Genet 1996; 98: 625-628.

[12] Barretina J, Taylor BS, Banerji S, Ramos AH, Lagos-Quintana M, Decarolis PL, Shah K, Socci ND, Weir BA, Ho A, Chiang DY, Reva B, Mermel CH, Getz G, Antipin Y, Beroukhim R, Major JE, Hatton C, Nicoletti R, Hanna M, Sharpe T, Fennell TJ, Cibulskis K, Onofrio RC, Saito T, Shukla N, Lau C, Nelander S, Silver SJ, Sougnez C, Viale A, Winckler W, Maki RG, Garraway LA, Lash A, Greulich H, Root DE, Sellers WR, Schwartz GK, Antonescu CR, Lander ES, Varmus HE, Ladanyi M, Sander C, Meyerson M and Singer S. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet 2010; 42: 715-721.

[13] Schulze JM, Wang AY and Kobor MS. YEATS domain proteins: a diverse family with many links to chromatin modification and transcription. Biochem Cell Biol 2009; 87: 65-75.

[14] Schmitt J, Fischer U, Heisel S, Strickfaden H, Backes C, Ruggieri A, Keller A, Chang P and Meese E. GAS41 amplification results in overexpression of a new spindle pole protein. Genes Chromosomes Cancer 2012; 51: 868-880.

[15] Zimmermann K, Ahrens K, Matthes S, Buerstedde JM, Stratling WH and Phi-van L. Targeted disruption of the GAS41 gene encoding a putative transcription factor indicates that GAS41 is essential for cell viability. J Biol Chem 2002; 277: 18626-18631.

[16] Gandhi SG, Bag I, Sengupta S, Pal-Bhadra M and Bhadra U. Drosophila oncogene Gas41 is

an RNA interference modulator that intersects heterochromatin and the small interfering RNA pathway. FEBS J 2015; 282: 153-173.

[17] Llanos S, Efeyan A, Monsech J, Dominguez O and Serrano M. A high-throughput loss-of-function screening identifies novel p53 regulators. Cell Cycle 2006; 5: 1880-1885.

[18] Park JH and Roeder RG. GAS41 is required for repression of the p53 tumor suppressor pathway during normal cellular proliferation. Mol Cell Biol 2006; 26: 4006-4016.

[19] Park JH, Smith RJ, Shieh SY and Roeder RG. The GAS41-PP2Cbeta complex dephosphorylates p53 at serine 366 and regulates its stability. J Biol Chem 2011; 286: 10911-10917.