Original Article
Long non-coding RNA AK001058 regulates tumor growth and angiogenesis in colorectal cancer via methylation of ADAMTS12

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Received July 28, 2019; Accepted August 2, 2019; Epub September 15, 2019; Published September 30, 2019

Abstract: Colorectal cancer, a common gastrointestinal malignant tumor, has been a leading cause of cancer related deaths. Long non-coding RNAs (lncRNAs) play an important role in regulating cancer development. The aim of this study was to investigate the role and potential mechanism of lncRNA AK001058 in colorectal cancer. To establish tumor xenografts, BALB/c nude mice received subcutaneously injection of SW480 cells with transfection targeting AK001058 (overexpression or knockdown). Tumor growth was observed and recorded. The relative gene expression levels were determined by quantitative real-time PCR or western blot. Cell apoptosis was determined by tunnel analysis. Microvessel morphology changes were detected by H&E staining. Methylation level of CpG island was analyzed using methylation specific PCR. The results showed that AK001058 overexpression notably accelerated tumor growth. AK001058 overexpression also decreased cell apoptosis, worsened microvessel morphology and increased the expression of VEGFA and angiopoietin II. Moreover, AK001058 decreased the expression of ADAMTS12 by increasing its methylation level. Nevertheless, AK001058 knockdown exerted the opposite function. Therefore, AK001058 knockdown could effectively inhibit tumor growth mostly accounting for decreased cell apoptosis and tumor angiogenesis, which was partly dependent on the high methylation level of ADAMTS12. These data provided a novel therapeutic strategy of colorectal cancer.

Keywords: AK001058, ADAMTS12, colorectal cancer, angiogenesis, methylation

Introduction
Colorectal cancer is a common gastrointestinal malignant tumor diagnosed both in females and males. It is a leading cause of cancer related deaths with an incidence of 134490 new cases and a mortality of 49190 deaths every year in United States [1]. In China, colorectal cancer ranks fifth among all malignant cancers [2]. In recent years, with the development of economy and living standard, great changes of structure of diet have taken place, and the incidence of colorectal cancer trends to be increased. Despite of the improvement of chemotherapy and surgical technic for treatment of clinical patients, the 5-year overall survival still remains unsatisfactory [3]. Many advanced colorectal cancer has a poor prognosis due to local recurrence and distant metastasis [4]. Therefore, an economic and reliable early detection is urgently needed to provide screening to patients suffering from colorectal cancer so as to improve their prognosis.

Long non-coding RNA (lncRNAs), longer than 200 bp, have received great concern as lncRNAs can regulate genome function and gene expression [5]. In recent years, lncRNAs have been found to play important roles in regulating colorectal cancer development. LncRNA-MALAT1 has been discovered in recurrent colorectal cancer and metastatic site in postsurgical patients, and further experiments found that MALAT1 was involved in the metastasis of colorectal cancer by regulating the transcriptional and translational levels of proto-oncogene RUNX2 [6]. LncRNA DANCR could promote cell migration and invasion through inhibition of lncRNA-LET in gastric cancer [7]. In our previous research, lncRNA AK001058 has been
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Demonstrated to be upregulated in colorectal cancer line cells. Moreover, knockdown of AK001058 could suppress cell proliferation, migration and invasion, which might partly depend on the methylation of ADAMTS12, a potential anti-oncogene located on chromosome 5. ADAMTS12 knockdown promoted cell proliferation and decreased adhesion between cells, thus promoting tumor cell metastasis [8]. However, the oncogene activity of AK001058 was only shown in vitro, in vivo experiments is also needed to perfect and further demonstrate this point.

Therefore, this study aimed to verify the function of AK001058 on tumorigenesis in vivo, and found its potential molecular mechanism of action, providing a feasible strategy for treatment of colorectal cancer.

Materials and methods

Establishment of tumor xenografts in mice

Male BALB/c nude mice (n = 6 for each group) were brought from Beijing Vital River Laboratory Animal Technology Co., Ltd, and were maintained under specific pathogen-free conditions. All mice studies were approved by the Institutional Animal Care and Use Committee of Taizhou First People’s Hospital.

For in vivo tumorigenicity, we subcutaneously inoculated 5 × 10⁶ SW480 cells with different AK001058 expressions (transfection control, overexpression, knockdown), which have been conducted in our previous work, into the flank of every mouse. The tumor volume of every mouse was measured every five days. Tumor volume = 1/2 (length × width²). At last, mice were sacrificed and the tumors were photographed, weighted, and fixed in 4% paraformaldehyde or stored in liquid nitrogen for further experiments.

Quantitative real-time PCR (qRT-PCR)

To gain total RNA, tumors were homogenized with TRIzol (Takara, Shiga, Japan). Total RNA was reverse-transcribed into cDNA and then subjected to qRT-PCR using FastStart Universal SYBR Green Master mix (Roche Diagnostics, Mannheim, Germany). GAPDH was used as an internal control. Relative gene expression was calculated using 2^{-ΔΔCt} method.

Western blot

To gain protein, tumors were homogenized with RIPA buffer (Millipore, MA, USA) containing a complete protease inhibitor tablet (Roche, Basel, Swiss). The concentration of protein in the supernatant was measured by Bradford relative protein quantification. The same amount of protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% non-fat milk for 2 h at room temperature, membranes were incubated with the primary antibodies at 4°C overnight. Subsequently, membranes were incubated with horse-radish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA). After washing, blots were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Amersham, UK).

Histological analysis

The microvessel morphology of mice suffering from tumors was assessed by hematoxylin-eosin (H&E) staining. After molding with paraffin, tissues were cut into 5-μm sections and stained with H&E. The histological changes were observed under a light microscopy.

Tunnel analysis

The apoptosis of tumor tissues was calculated using TUNEL assay kit (KeyGEN BioTECH, Jiangsu, China) in accordance with the protocol of manufacturer. In brief, the 5-μm tissue sections were dewaxed, hydrated, incubated with a methanol solution containing 0.2% H₂O₂ for 15 min to block endogenous peroxidase activity and incubated in proteinase K working solution for 30 min. After washing with PBS, all sections were incubated with TUNEL mixture for 60 min, followed by an incubation with DAPI for 10 min. Finally, the tissue sections were observed with a confocal microscopy.

Methylation analysis

DNA was extracted from tumors using a DNeasy Tissue Kit (Qiagen, Chatsworth, CA) in accordance with the protocol of manufacturer. The methylation level of ADAMTS12 gene promoter was determined using methylation specific PCR (MSP) according to the EZ DNA Methylation Lighting Kit. The sequence of methylated and demethylated primers are shown as followed:
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forward 5'-GAGTTTGGGAGGAAGATGTATT-3', reverse 5'-CTAACAATATCCACTTTCAACAAAA-3' for methylated template and forward 5'-GAGTTTCGGGAGGAAGATGTATC-3', reverse 5'-ACAATATCCGCTTTCGACG-3' for demethylated template. The amplified products were placed in 20 g/L agarose gel electrophoresis, and the bands were visualized under ultraviolet irradiation.

Statistical analysis

All data were presented as mean ± SD. All statistical analysis were performed using SPSS (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Prism Software, Inc., San Diego, CA, USA) using the one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

AK001058 influenced tumor growth in colorectal cancer mice

First, we evaluated the biological effects of AK001058 on colorectal tumorigenesis in a xenograft mouse model. The colorectal cancer cell line SW480 cells with different AK001058 expressions were subcutaneously injected into the flank of every mouse. We could observe the tumor size and weight under different groups (Figure 1A-C). The tumor in AK001058 group was largest and heaviest, and the tumor in siRNA group was smallest and lightest among groups, suggesting that AK001058 obviously promoted tumor growth, while knockdown of AK001058 could notably inhibit tumor growth. Besides, the growth curves of tumors indicated...
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that the tumor size and volume became larger after cell implantation (Figure 1D). In AK001058 group, the tumor grew fastest, promoting tumor growth, while in siRNA group, tumor in mice grew slowest, indicating an obvious inhibition of tumor growth when AK001058 was knockdown.

**AK001058 regulated tumor cell apoptosis in tissues**

qRT-PCR demonstrated that mRNA level of AK001058 was significantly upregulated in mice injected with cells that AK001058 was overexpression, and mRNA level of AK001058 was significantly downregulated in mice injected with cells that AK001058 was knockdown (Figure 2A). Then, we hypothesized that since AK001058 accelerated tumor growth in mice, cancer cells of tumor tissue in AK001058 group might also grow well and have little apoptosis. The results in Figure 2B demonstrated this hypothesis. Tunnel analysis showed that tumor tissue has little cell apoptosis in AK001058 group, and more cell apoptosis in siRNA group, indicating that AK001058 knockdown inhibited cell apoptosis in tumor tissue.

**AK001058 regulated tumor angiogenesis in vivo**

To further investigate the mechanism of action, we evaluate the effect of AK001058 on angiogenesis. H&E staining was carried out to observe the microvessel in tissues. The results in Figure 3A showed that microvessel morphology changed. More and larger microvessel was found in AK001058 group, and the microvessel was smaller in siRNA group. Vascular endothelial growth factor A (VEGFA) and angiopoietin (Ang II) are important proangiogenic cytokines that sustain tumor angiogenesis [9]. The results in Figure 3B showed that protein expressions of VEGFA and Ang II were increased in AK001058 group and decreased in siRNA group. These results suggest that overexpression of AK001058 significantly promote tumor angiogenesis, and knockdown of AK001058 could inhibit tumor angiogenesis.

**AK001058 regulated ADAMTS12 by modulation of methylation level**

Finally, we detected the expression of ADAMTS12 and its methylation level to find out more potential mechanism about AK001058 in cancer. The results showed that in AK001058 overexpression group, protein expression and mRNA level of ADAMTS12 was obviously downregulated, and in AK001058 knockdown group, protein expression and mRNA level of ADAMTS12 was obviously upregulated (Figure 4A, 4B). In addition, methylation-specific PCR (MSP) was performed and the results showed that overexpression of AK001058 increased methylation level of ADAMTS12, while AK001058 knockdown inhibited methylation level of ADAMTS12 CpG islands (Figure 4C), indicating that AK001058 might regulate the expression of ADAMTS12 by modulating its CpG islands methylation.
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Discussion

In this study, several novel findings have been discovered. Firstly, we demonstrated the influence of AK001058 in vivo on tumor growth, and knockdown of AK001058 was beneficial to inhibit tumor growth in mice. Then, to explore the potential mechanism of AK001058, which was closely involved in the cancer development in mice, we detected the cell apoptosis and tumor angiogenesis under the changes of AK001058. Overexpression of AK001058 was able to promote tumor cell apoptosis and tumor angiogenesis, effectively leading to a decline of tumor growth. Finally, decreased expression of ADAMTS12 and promoter hypermethylation of ADAMTS12 were discovered when AK001058 was overexpressed. Thus, AK001058 was considered to influence tumor growth through regulating cell apoptosis and tumor angiogenesis targeting methylation of ADAMTS12.

Tumor angiogenesis, one of the main characters of cancer cells, is necessary for tumor progression. Vascular network around tumor provided oxygen and nutrient substance, stimulating tumor growth and metastasis [10]. Meanwhile, increased angiogenic factors in tis-

Figure 3. AK001058 regulated tumor angiogenesis in vivo. In different groups with different expression level of AK001058, microvessel morphology changes were observed using H&E staining (A). Then, the protein expressions of VEGFA and angiopoietin II were determined by western blot and the protein bands were quantified (B). Data was presented as mean ± SD. **, ***P<0.01, 0.001 vs NC.

Figure 4. AK001058regulated ADAMTS12 by modulation of methylation level. The protein expression of ADAMTS12 was determined by western blot and the protein bands were quantified (A). The mRNA level of ADAMTS12 was determined by quantitative real-time PCR (B). The methylation level of ADAMTS12 CpG sites was detected by Methylation-specific PCR (C). Data was presented as mean ± SD. ***P<0.001 vs NC.
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sues reflected the aggressiveness of tumor cells and stimulated tumor growth and metastasis [11]. In cancer, the pro- and anti-angiogenic factors are out of balance, followed with uncontrolled angiogenesis. Therefore, anti-angiogenic therapies have been approved for cancer treatment [12, 13]. Many potential drug for cancer therapy exhibited anti-tumor effects by inhibiting tumor angiogenesis, such as miRNA-497 and baicalein [14, 15]. VEGFA, a member of VEGF family, play an important role in tumor angiogenesis and tumor growth [16]. Ang II, an important member of Ang family, is also critical in tumor vascular regulation. High expressions of VEGFA and Ang II were closely involved in worsened tumors, and inhibition of VEGFA and Ang II was beneficial in the tumor therapy [9]. In the present study, microvessel density was increased under high expression of AK001058, as well as a high expression of VEGFA and Ang II, indicating a stronger angiogenesis was generated under high expression of AK001058. Thus overexpression of AK001058 could promote tumor angiogenesis, and then contributed to the tumor growth. Therefore, inhibition of AK001058 would be a promising potential treatment for colorectal cancer through inhibiting tumor angiogenesis.

ADAMTS12 has been demonstrated to exhibit anti-tumorigenic property in previous studies [8]. As a novel anti-tumor protease that can reduce the proliferative properties of tumor cells, epigenetic silence of ADAMTS12 in tumor cells play an important role in controlling the progression of cancer [17]. Besides, ADAMTS12 also inhibited tumor development in colorectal cancer. Meanwhile, patients with ADAMTS12 expression exhibited better prognosis than those without ADAMTS12 expression, indicating that ADAMTS12 could be considered as a diagnostic and prognostic marker for colorectal cancer [18], and could become a therapeutic target for the treatment of colorectal cancer. In the present study, overexpression or knockdown of AK001058 directly influenced the expression of ADAMTS12. The high expression of ADAMTS12 in siRNA group contributed to an inhibition of tumor development, which might well explain the reason that tumor growth was inhibited in siRNA group, indicating that knockdown of AK001058 suppressed tumor growth through improving the expression of ADAMTS12. However, how ADAMT12 worked to influence tumor development is still a question and is deserved to be investigated. DNA methylation is a major epigenetic modification that is strongly involved in cancer diagnosis [19]. Promoter hypermethylation was an important mechanism of critical genes silence. ADAMTS12 gene promoter is reported to be hypermethylated in primary colorectal tumors and colorectal cancer cell lines, and the promoter methylation of ADAMTS12 silenced its gene expression [17]. Similarly, methylated ADAMTS12 was detected in AK001058 group with lower expression of ADAMTS12 in the present study. Interestingly, high expression of ADAMTS12 in cancer cells have a beneficial protective effect toward tumor growth and invasion. ADAMTS12-deficiency accelerated the angiogenic response and tumor invasion both in vivo and in vitro, meaning that ADAMTS12 was closely associated with angiogenesis [20]. In the present study, knockdown of AK001058 significantly changed microvessel morphology and decreased protein expression of VEGFA and Ang II, meaning a decline of angiogenesis. Therefore, we can conclude that knockdown of AK001058 inhibited tumor growth and angiogenesis through promoter hypermethylation of ADAMTS12.

Conclusion

In a summary, we discovered a potential target, AK001058, for colorectal cancer treatment. In this study, we not only verified its function on regulating tumor growth in vivo, but also disclosing its potential mechanism of action in tumor progression. The results above suggested that knockdown of AK001058 could effectively inhibit tumor growth mostly accounting for decreased cell apoptosis and tumor angiogenesis, which was partly dependent on the high methylation level of ADAMTS12. These data provided a novel and promising idea for the therapeutic strategy of colorectal cancer.

Acknowledgements

This study is supported by Medical Science and Technology Program of Zhejiang Province (No. 2018KY896) and Scientific Research Project of Taizhou Science and Technology Bureau in Zhejiang Province (NO. 162yw05).

Disclosure of conflict of interest

None.
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