RESEARCH ARTICLE

hARIP2 is a Putative Growth-promoting Factor Involved in Human Colon Tumorigenesis

Rui-Feng Gao[1,2&], Zhan-Dong Li[3&], Jing Jiang[1&], Li-Hua Yang[1], Ke-Tong Zhu[3], Rui-Xin Lin[4], Hao Li[3]*, Quan Zhao[1]*, Nai-Sheng Zhang[2]*

Abstract

    Activin is a multifunctional growth and differentiation factor of the growth factor-beta (TGF-β) superfamily, which inhibits the proliferation of colon cancer cells. It induces phosphorylation of intracellular signaling molecules (Smads) by interacting with its type I and type II receptors. Previous studies showed that human activin receptor-interacting protein 2 (hARIP2) can reduce activin signaling by interacting with activin type II receptors; however, the activity of hARIP2 in colon cancer has yet to be detailed. *In vitro*, overexpression of hARIP2 reduced activin-induced transcriptional activity and enhanced cell proliferation and colony formation in human colon cancer HCT8 cells and SW620 cells. Also, hARIP2 promoted colon cancer cell apoptosis, suggesting that a vital role in the initial stage of colon carcinogenesis. *In vivo*, immunohistochemistry revealed that hARIP2 was expressed more frequently and much more intensely in malignant colon tissues than in controls. These results indicate that hARIP2 is involved in human colon tumorigenesis and could be a predictive maker for colon carcinoma aggressiveness.

Keywords: Activin - activin receptor II - human activin receptor - interacting protein 2 - colon tumorigenesis

Introduction

    Activin is a member of growth factor-beta (TGF-β) superfamily of extracellular signaling proteins. It has numerous regulatory functions such as cell proliferation and differentiation, apoptosis, metabolism, homeostasis, immune responses, wound repair and various endocrine functions through signaling mediated by type I and type II serine/threonine kinase receptors (Bao et al., 2005; Chen et al., 2006). Activin also binds directly to the type II receptor, leading to the recruitment, phosphorylation and subsequent activation of the type I receptor. Upon activation, the type I receptor will bind to and phosphorylates a subset of cytoplasmic Smad proteins. These phosphorylated Smadswill be translocated to the nucleus which controls the transcription of target genes (Panopoulou et al., 2002).

    So far, known functions of activin type II receptors (ActRIIs) are limited to ligand binding, type I receptor recruitment, and transphosphorylation. ActRIIs include two subtypes of activin type II receptors: type IIA (ActRIIA) and IIB (ActRIIB), each of which is encoded by unique genes. Activin has also been shown to inhibit the proliferation of a variety of tumor cell types. Studies demonstrated that activin has been associated with colon cancer (Deacu et al., 2004; Bauer et al., 2012).

    As known, human activin receptor-interacting protein 2 (hARIP2) can enhance ActRII endocytosis and reduce ActRIIA receptor expression on cell membranes via Ral/RalBP1-depending pathway, and has a capability of suppressing activin-induced signal transduction (Han et al., 2011; Hinck et al., 2012). Therefore, we hypothesis ARIP2 may involve in the functional regulation of human colon cancer.

    In the present study, we investigated hARIP2 effect on activin signal transduction, cell proliferation, and colony formation in colon cancer cells. The results showed that overexpression of hARIP2 decreased activin-induced transcriptional activity and promoted cell proliferation and colony formation. Also, hARIP2 promoted colon cancer cell apoptosis. Furthermore, the cellular distribution of hARIP2 was characterized in human colon tissues. Immunohistochemical observation from colon cancer patients revealed that the hARIP2 levels were significantly higher in colon cancer tissues than benign colon tissues. hARIP2 plays a crucial role in colon carcinogenesis.

Materials and Methods

Regents

    The specific antisera were purified by passing through protein A Sepharose (Amersham Biosciences) and used

[1]Changchun University of Science and Technology, [2]College of Veterinary Medicine, Jilin University, [3]College of Biology and Food Engineering, Jilin Teachers Institute of Engineering and Technology, [4]The Second Hospital of Jilin University, Changchun, China [&]Equal contributors *For correpsondence: nszhang9@sina.com, hltiet@163.com, rumeng2215137@163.com

for immunohistochemical analysis. The anti-FLAG (M2) antibody was purchased from Sigma and anti-GAPDH antibody was purchased from Abcam Company (UK). Lipofectamine 2000 cell transfection was purchase from Invitrogen (USA).

Plasmids Construction

To create the hARIP2 RNA interference (RNAi) expression vector, the pSilencer4.1-CMV neo Vector (Ambion, USA) was used for DNA vector-based small interfering RNA (siRNA) synthesis. hARIP2 siRNA corresponds to nucleotides 516 to 535. The sequences of the synthesized oligonucleotides are: 5'-GATCCCGCCAGAAAGCCTT AAGACTATTCAAGAGATAGTCTTAAGGCTTTC TGGTTTTTTGGAAA-3'(forward) and 5'-AGCT TTTCCAAAAAACCAGAAAGCCTTAAGACTATC TCTTGAATAGTCTTAAGGCTTTCTGGCGG-3' (reverse).

The paired oligonucleotides were annealed in buffer (100 mmol/L Kacetate, 30 mmol/L HEPES-KOH (pH 7.4), and 2 mmol/L Mg acetate). The mixture was first incubated at 90°C for 3 minutes, then 37°C for 1 hour. The oligonucleotide containing terminal BamHI-HindIII sites was subcloned into the BamHI-HindIII sites of the pSilencer4.1-CMV neo vector to generate the pSilencer4.1-CMV neo-hARIP2 siRNA vectors. A negative control scrambled siRNA (Ambion, USA), which has no significant homology to mouse or human gene sequences, was designed to detect any nonspecific effects.

Cell Culture and Transfection

Human colon cancer cells HCT8 and SW620 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). The cells were transfected with the indicated plasmids using Lipofectamine 2000 cell transfection reagent according to the manufacturer's protocol.

Activin-responsive Promoter Assay

The CAGA-lux construct has been described previously (Busanadiego et al., 2013). CAGA-lux, cytomegalovirus β-galactosidase, and pcDNA3, pcDNA3-FLAG-hAIRP2, control siRNA or hARIP2 siRNA were introduced into HCT8 cells and SW620 cells using Lipofectamine 2000 cell transfection reagent according to the manufacturer's protocol. Stimulation by activin (50 ng/mL) and measurement of luciferase activity using FLUOstar OPTIMA (BMG LABTECH, Germany) were performed as described previously (Yoom et al, 2013).

dWestern Blot Analysis

HCT8 cells and SW620 cells were transfected with an indicated plasmids using Lipofectamine 2000 cell transfection reagent (Invitrogen, USA) according to the manufacturer's protocol. Two days after transfection, HCT8 cells and SW620 cells were washed three times in PBS, followed by lysis in a lysis buffer [50 mM Tris-HCl (pH=7.5), 150 mM NaCl, 1 mM NaF, 1% Nonidet P-40,

1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin]. The lysate was centrifuged and the protein concentration of each supernatant was determined by Coomassie protein assay reagent (Beyotime Biotechnology, Jiangsu, China). Equal amounts of proteins were then separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Then the membranes were incubated with anti-GAPDH and anti-hARIP2 antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The specific proteins were detected by ECL (Piece Biotechnology, USA).

Cell Proliferation Analysis

HCT8 cells and SW620 cells were transfected with pcDNA3 (control), pcDNA3-FLAG-hARIP2, control siRNA, and hARIP2 siRNA using the Lipofectamine 2000 cell transfection reagent (Invitrogen, USA). 24h after transfection, the cells were seeded in triplicate in 96-well plates at a density of 1×10^4 cells per well with DMEM medium (Gibco) containing 10% FBS. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were done at 24, 48, 72 and 96 h. Twenty microliters of MTT stock solution (5 mg/mL in PBS, Sigma) were then added to each well and the plates were further incubated for 4h. After incubation, 150 μL of dimethyl sulphoxide was added to each well, mixed by vigorous pipetting, and incubated for 15 minutes at 37°C. Absorbances were measured immediately at 570 nm.

Clonogenic Assay

The effects of hARIP2 on the proliferation of HCT8 cells and SW620 cells were assessed by the colony formation assay which was performed as described (Zhang et al., 2013). Briefly, HCT8 cells and SW620 cells were transfected with control, hARIP2, control siRNA, or hARIP2 siRNA for 24h and collected by trypsinization. The cells were counted and seeded into 6-well plates with 1000 cells/well. The cells were cultured for 14 days with growth media being replaced every 3 days. The cells were then stained with 0.5% crystal violet (in methanol-water, 1:1) and counted under the microscope (Olympus, Japan). An aggregate composed of more than 50 cells was recognized as a colony.

Cell Apoptosis Analysis

To determine the effects of hARIP2 on the proliferation of HCT8 cells and SW620 cells apoptosis, 5×10^5 colon cancer cells were seeded in 6-well plates and transfected with control, hARIP2, control siRNA, or hARIP2 siRNA for 48h. Then the colon cancer cells were harvested, fixed in 70% ethanol, washed in phosphate-buffered saline (PBS), resuspended in 1 mL of PBS containing 1 mg/mL RNase and 50 mg/mL propidium iodide, incubated in the dark for 30 minutes at room temperature, and analyzed by flow cytometer (BD, USA). The data were analyzed using MultiCycle software.

Immunohistochemical Analysis of hARIP2 Expression in Colon Cancer Tissues

Paraffin embedded colon cancer tissue sample blocks

were collected from the Department of Pathology and Pathophysiology of Jilin University. Tissue materials included 50 cases of grade I colon cancer tissue, 32 cases of grade II colon cancer tissue, 12 cases of grade III colon cancer tissue. Patient information and tumor pathology have been summarized in Table 1. Immunohistochemical images of the sections were captured under a microscope and photographed. Immunoreactivity was scored on an ordinal scale from 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining) for the protein of hARIP2 evaluated. In order to diminish interobserver variability, all of the cases were scored in a blinded fashion.
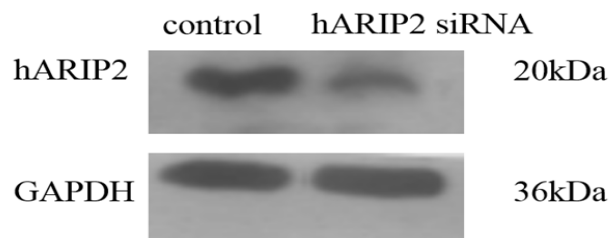
For immunohistochemistry assays, colon cancer tissues were cut into 5 μm thick sections and mounted on poly-(L-lysine)-coated slides. After deparaffinization and epitope retrieval in 10 mM citrate buffer, the non-specific binding sites were blocked by incubating the section in 5% normal rabbit serum in PBS (pH 7.4) for 15-20 minutes. After washing in PBS, the sections were incubated with the primary antibody anti-hARIP2 at a 1:500 dilution in PBS/1% BSA for 12h at 4°C or mouse IgG as control. After extensive washing with PBS, the biotinylated secondary antibody was added onto the sections and incubated at room temperature for 20 minutes. After washing in PBS, the sections were incubated with a peroxidase conjugated avidin-biotin complex for 20 minutes at room temperature. Then the reaction product was developed using 3, 3-diaminobenzidine tetrahydrochloride and hydrogen peroxide in PBS. Sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in histosol and coverslips mounted using neutral balsam.

Statistical Analysis

All *in vitro* experiments were repeated at least three times. Each result was the reflection of the mean of three independent experiments. The statistical significance of the differences between groups' analyses was determined by the Student's t-test. Comparisons resulting in a *p* value of less than 0.05 were considered statistically significant and identified in the figures with an asterisk (*).

Results
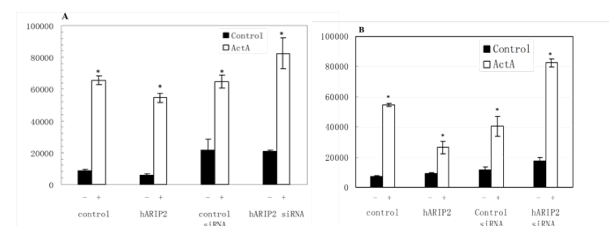
RNA Interference Inhibits the Expression of hARIP2

To study the role of hARIP2 in colon cancer cells, we used RNAi strategy to down-regulate the molecule. In HCT8 cells, the expression of hARIP2 level was partly inhibited after transfection with hARIP2 siRNA comparing with Control siRNA (Figure 1). Parallel results were obtained in SW620 cells (data not shown). These results indicate that siRNA in hARIP2 specifically inhibited hARIP2 expression in colon cancer cells in part.

The Effect of hARIP2 on Activin-induced Transcriptional Response

HCT8 cells and SW620 cells were transfected with either pcDNA3, pcDNA3-ARIP2, control siRNA, or ARIP2 siRNA together with a reporter plasmid, CAGA-lux, and activin-induced luciferase activity was measured. Overexpression of hARIP2 in HCT8 cells and SW620 cells decreased activin-induced transcriptional activity (Figure 2). In contrast, inhibiting hARIP2 expression through RNA interference increased activin-induced transcriptional activity (Figure 2). These data suggest that hARIP2 has an inhibitory effect on activin-induced transcriptional response.

Effect of hARIP2 on Colon Cancer Cell Proliferation

To examine the roles of hARIP2 in human colon tumor cell proliferation, we transfected HCT8 cells and SW620 cells with pcDNA3, pcDNA3-FLAG-hARIP2, control siRNA or hARIP2 siRNA. Proliferation activities shown in Figure 3 were the means of three independent experiments. We observed that overexpression of hARIP2 increased

Table 1. Characteristics of Colon Tumors

Characteristics		Data
Lymph node status, n (%)	Negative	40 (42.6)
	Positive	54 (57.4)
	Unknown	0 (0)
Tumor size, n (%)	0-2 cm	28 (29.8)
	2-5 cm	56 (59.6)
	>5 cm	10 (10.6)
Tumor grade, n (%)	Grade 1	50 (53.2)
	Grade 2	32 (34.0)
	Grade 3	12 (12.8)

Figure 1. RNA Interference Inhibits the Expression of hARIP2. HCT8 cells were transfected with negative control (control siRNA), pSilencer-hARIP2(hARIP2 siRNA) and analyzed by western blotting. Equal amounts of cell lysates were analyzed by western blotting with anti-GAPDH and affinity-purified anti-hARIP2 antibodies


Figure 2. Effect of hARIP2 on activin-induced transcription. pcDNA3-hARIP2 and hARIP2 siRNA have opposite effects on activin-induced transcription in HCT8 cells and SW620 cells. (A) HCT8 cells were transfected with CAGA-lux, cytomegalovirus-β-galactosidase, and either control, pcDNA3-hARIP2, control siRNA or hARIP2 siRNA. (B) SW620 cells were transfected with CAGA-lux, cytomegalovirus-β-galactosidase, and either control, pcDNA3-ARIP2, control siRNA or hARIP2 siRNA then incubated with 50 ng/mL activin A for 24h. The level of luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and SD of triplicate determinations (*p<0.05 vs activin-treated cells with control or control siRNA by using a t-test)
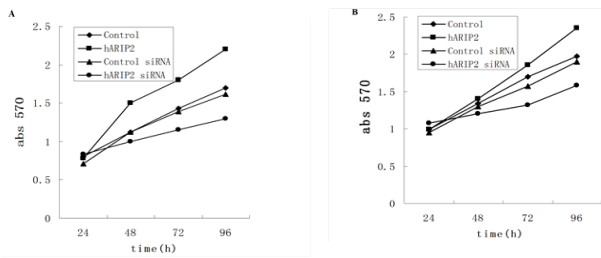
Figure 3. *In vitro* **effects of hARIP2 on the Proliferation of HCT8 Cells and SW620 Cells.** (A) growth curves for control, hARIP2, control siRNA and hARIP2 siRNA-transfected cells in vitro proliferation assays for HCT8 cells by MTT assay. (B) growth curves for control, hARIP2, control siRNA and hARIP2 siRNA-transfected cells in vitro proliferation assays for SW620 cells by MTT assay. MTT assays were done at 24, 48, 72, and 96h. The absorbances were measured immediately at 570 nm. Each data is expressed as the mean±SD obtained from triplicate experiments
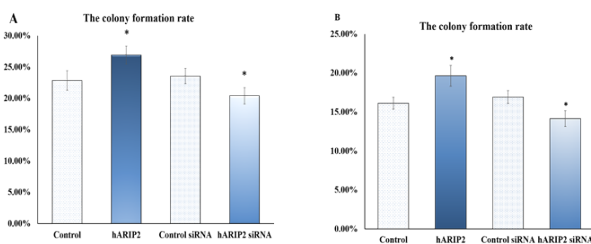
Figure 4. Effects of hARIP2 on Colony Formation in HCT8 Cells and SW620 Cells. The cells transfected with control, hARIP2, control siRNA and hARIP2 siRNA, respectively, were cultured for 14 days. Formed colonies were counted under the microscope after staining with 0.5% crystal violet. An aggregate consisting of more than 50 cells was counted as a colony. (A) the colony formation rate of HCT8 cells transfected with control, hARIP2, control siRNA and hARIP2 siRNA, respectively. (B) the colony formation rate of SW620 cells transfected with control, hARIP2, control siRNA and hARIP2 siRNA, respectively. Each data is expressed as the mean±SD obtained from triplicate experiments (*$p<0.05$ *vs* control or control siRNA by using a t-test)

cell proliferation, and inhibiting hARIP2 expression by RNA interference decreased cell proliferation from MTT analysis. Similar results were obtained in other tumorous cells. These results indicate that hARIP2 enhanced human colon tumor cell proliferation.

*Effect of hARIP2 on Tumor Cell Colony Formation*

Next, we wanted to evaluate the effect of hARIP2 on the reproductive potential of a single cell in colony formation assay. We transfected HCT8 cells and SW620 cells with pcDNA3, pcDNA3-FLAG-hARIP2, control siRNA or hARIP2 siRNA. As shown in Figure 4, overexpression of hARIP2 increased cell colony formation ability and inhibition of hARIP2 expression by RNA interference decreased cell proliferation. These results suggest that hARIP2 stimulates colony formation in HCT8 cells and SW620 cells.

Effect of hARIP2 on Cell Apoptosis

To confirm the effect of the new factor on cell apoptosis in colon cancer cells, Cell apoptosis was evaluated using flow cytometric analysis in HCT8 cells and SW620 cells. When the cells were transfected to either pcDNA3-FLAG-
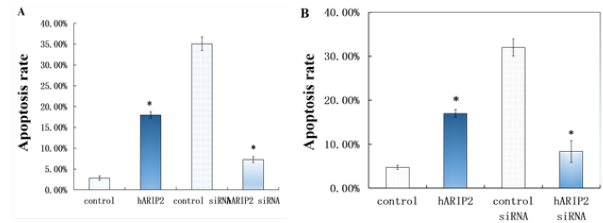
Figure 5. The Effect of hARIP2 on Cell Apoptosis in Colon Cancer Cells. The cell apoptosis was assessed by flow cytometry. The changes were shown. (A) the HCT8 cells were transfected with control, FLAG-pcDNA3-hARIP2, control siRNA and hARIP2 siRNA respectively. (B) the SW620 cells were transfected with control, FLAG-pcDNA3-hARIP2, control siRNA and hARIP2 siRNA respectively.The asterisk indicates a significant difference between control and FLAG-pcDNA3-hARIP2, control siRNA and hARIP2 siRNA respectively as analyzed by a t-test, $p<0.01$
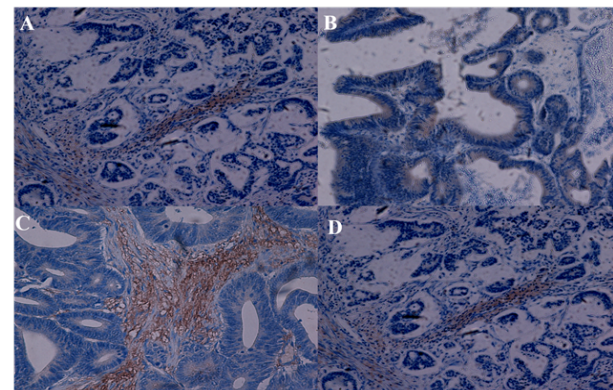
Figure 6. hARIP2 Immunostaining in Malignant and Normal Tissues. Tissue stained brown is positive for immunoreactivity. Nuclei are stained blue. (A) negative control treated without primary antibody. (B) grade I tissues. (C) grade II tissues. (D) grade III tissues. Original magnification is ×20 for all panels

Table 2. Correlations for Intense hARIP2 and Clinical and Pathological Grade of Colon Cancer

Tissue	0,1 (No or Weak)	hARIP2 Staining Intensity 2,3 (Moderate or Strong)	Positive rate
Grade 1 tissue	25	25	50%
Grade 2 tissue	3	29	90.32%
Grade 3 tissue	0	12	100%

Table 3. Correlation for intense hARIP2 and Lymph Mode Status of Colon Cancer

Lymph node status	0,1 (No or Weak)	hARIP2 Staining Intensity 2,3 (Moderate or Strong)	Positive rate
0-3	16	58	78.38%
4/9/14	0	8	100%
≥10	0	12	100%

Table 4. Correlation for Intense hARIP2 and Tumor Size of Colon Cancer

Lymph node status	0,1 (No or Weak)	hARIP2 Staining Intensity 2,3 (Moderate or Strong)	Positive rate
<2Cm	12	14	58.85%
2-5Cm	5	53	91.38%
>5Cm	0	10	100%

hARIP2 or control, the result showed that hARIP2 induces cell apoptosis significantly. However, when the cells were transfected with control siNRA and hARIP2 siRNA respectively, the result indicates that siRNA in hARIP2 reduces cell apoptosis (Figure 5).

Distribution of hARIP2 in Colon Cancer Tissues

hARIP2 was detected in colon cancer tissues. Omission of the primary antibody was used as a negative control (Figure 6A). Moderate to low staining levels were found in grade I colon cancer tissue (Figure 6B). Intense staining levels were detected in colon cancers including 32 cases of grade II colon cancer tissue, 12 cases of grade III colon cancer tissue (Figure 6C-D). A statistical analysis of the intensity scores for hARIP2 immunoreactivity indicated that hARIP2 was more frequently and much more intensely expressed in high grade colon cancer tissues (90.32% grade II colon cancer tissue; and 100% grade III colon cancer tissue; Table 2) than it was in low grade colon cancer tissues (50% grade I colon cancer tissue; Table 2). Furthermore, our result shows that the abundance of hARIP2 is proportional to the presence of lymph-node metastasis and tumor size in colon cancer (Table 3, Table 4).

Discussion

Colon cancer is one of the leading causes of cancer-related mortality worldwide (Pandurangan et al., 2013). The risk of developing colon polyps and cancer appears to be a result of the effect of genetic and environmental factors that promote the formation of adenomas and/or the progression of these adenomas to cancer (Zhi et al., 2007; Salimzadeh et al., 2011). These environmental and genetic factors contribute to colorectal cancer formation by promoting the accumulation of gene mutations and epigenetic alterations in colon epithelial cells.

Li et al. (2009) reported hARIP2 encoding sequence was cloned from normal human placenta tissue, composed of 558 bp, encodes a protein of 182 amino acids, and contains a single PDZ domain in its NH2-terminal region. However, hARIP2 did not show interaction with either TGF-β type II receptor or bone morphogenetic protein type II receptor. These results suggest that hARIP2 may have specific roles in activin signaling. Therefore, we further investigated the effect of hARIP2 on activin-induced transcriptional response in colon cancer cells. Our results showed that overexpression of hARIP2 in HCT8 cells and SW620 cells decreased activin-induced transcriptional activity and inhibition of hARIP2 expression through RNA interference increased activin-induced transcriptional activity. These data suggest that hARIP2 has an inhibitory effect on activin-induced transcriptional response in colon cancer cells.

Since hARIP2 can inhibit activin-induced transcription response and promote breast cancer cell, we speculate that hARIP2 may have an effect on colon cancer cell proliferation. Consequently, we overexpressed hARIP2 in HCT8 cells and SW620 cells and measured cell proliferation. hARIP2 can slightly induce colon cancer cells proliferation from MTT analysis, which is consistence with hARIP2 specific siRNA expression. Those results suggested that hARIP2 may be partially involved in colon tumorigenesis and tumorous development. In contrast, ectopic overexpression of hARIP2 (or its siRNA) on proliferation and colony formation of HCT8 cells and SW620 cells in culture were relatively modest.

Interestingly, overexpression of hARIP2 in colon cancer can induce cell apoptosis in our study.markedly. Thus, we suggest that hARIP2 promotes colon cancer cells proliferation and cell apoptosis at the same time, which maybe hARIP2 plays a crucial role in the initial stage of colon carcinogenesis. Although the potential mechanism for hARIP2-induced cell apoptosis is obscure from this study, we cannot rule out activin signal will involve in.

A recent study revealed that activin receptors and its signaling molecules are involved in human colon cancer cell lines (Deacu et al., 2004). Once there is the loss or reduction of activin signaling components, the colon cancer becomes more aggressive (Grau et al., 2006). However, distribution of hARIP2 in human colon cancer tissues is still unclear.

Hence, we collected human colon cancer tissue samples to investigate the expression of hARIP2 in the colon tissues in the present study. hARIP2 was more frequently and much more intensely expressed in high grade colon cancer tissue than it was in low grade colon cancer tissue and a significant association between hARIP2 staining and the presence of lymph-node metastasis in colon cancer. These results suggested that hARIP2 is involved in colon tumor metastasis. Likewise, our result shows that the abundance of hARIP2 is proportional to tumor size. Briefly, the colon cancer stage correlation with hARIP2 is intriguing.

In summary, we have characterized hARIP2's involvement in colon cancer. Further study disclosed that hARIP2 enhances cell proliferation, colony formation and cell apoptosis in HCT8 cells and SW620 cells. Immunohistochemistry results indicate that hARIP2 was more intensely expressed in high grade colon cancer tissues than in low grade cases.

Currently, the development of clinical applications for the members of the activin and its antagonists were intriguing (Bao et al., 2005). Studies showed that the efficacy of these factors in animal disease models might be beneficial in treating human diseases (Wankell et al., 2001; Zimmers et al., 2002). Based on above statement, hARIP2 might even represent a potential target for treatment of human colon cancer.

Acknowledgements

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