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Activin A as a novel biomarker for colorectal adenocarcinoma in humans

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Abstract. – OBJECTIVE: Early diagnostics of colorectal cancer is complicated by the lack of reliable serum biomarkers. This study aimed to investigate if the serum level of activin A can be used for diagnostics of this disease.

PATIENTS AND METHODS: In this study we measured the level of activin A in patients with colorectal adenocarcinoma, benign colorectal polyps, as well as in healthy subjects.

RESULTS: We found that the level of activin A was significantly higher in patients with colorectal adenocarcinoma, as compared to patients with polyps and healthy controls. Furthermore, activin A levels correlated well with the stage of colorectal cancer. The level of activin A was substantially reduced in post-operative patients. Immunohistochemical staining demonstrated that high levels of activin A were present in the adenocarcinoma tissue samples but not in the non-cancerous samples. RT-PCR further confirmed that mRNA of ßA subunit of activin A is significantly over-expressed in the majority of cancerous samples. Western blotting results further demonstrated the elevated level of activin A in cancer samples.

CONCLUSIONS: Taken together, the findings suggest that colorectal adenocarcinomas directly secret activin A into the blood stream. Measuring the serum level of activin A might be used as a reliable diagnostic and screening tool in clinical practice.

Key Words:
Activin A, Colon cancer, Colorectal adenocarcinoma, Cancer biomarkers.

Introduction

Colorectal cancer (CRC) is the third most common human malignancy and also one the leading causes of cancer-related mortality worldwide[1]. Adenocarcinoma is the most common histological type of colorectal cancer. The screening and early diagnostics of colorectal adenocarcinoma are crucial for successful treatment and favorable prognosis[2]. Conventional colonoscopy is considered to be the primary technique for colorectal adenocarcinoma screening programs. However, the use of colonoscopy is limited by a number of drawbacks such as complication rate, pain and patient's embarrassment. Non-invasive techniques for screening may provide good alternative to conventional colonoscopy. Even though the screening for serum carcinoembryonic antigen (CEA) is commonly practiced in clinical setting, this marker is not sufficient for diagnosis and staging of colorectal adenocarcinoma[3]. Finding a novel and reliable serum cancer marker is of great significance for screening and early diagnosis of this disease.

Activin, a member of the TGF-β superfamily, was originally described[4] for its ability to regulate the follicle stimulating hormone (FSH) secretion from the anterior pituitary gland. Activins were subsequently identified in many tissues including the gonads and extragonadal organs, such as adrenal gland, liver and placenta. Three activins have been identified in human tissues: activin A (βAβA), activin B (βBβB), and activin AB (βAβB), of which activin A is the most studied. Activin A has various functions in regulating the cell differentiation, proliferation, apoptosis and carcinogenesis[5]. It has been shown that activin A plays roles in the development and progression of breast carcinomas, ovary carcinomas, as well as colorectal carcinoma[6-8]. Previous studies have revealed that activin A is over-expressed in human colorectal tumors, especially at stage IV of disease[9], raising the possibility that activin A may play a role in the progression of disease to advanced stages. However, limited studies have

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been conducted on the colorectal adenocarcinoma patients' serum level of activin A, which is potentially a novel marker in screening for this malignancy. In this study, the serum level of activin A has been assayed in the peripheral blood of colorectal adenocarcinoma patients, as well as in the blood of patients with benign colorectal polyps and healthy subjects for comparison. Furthermore, we investigated the expression of activin in colorectal adenocarcinoma tissues by RT-PCR, immunohistochemical staining and Western blotting. The role of activin in the proliferation of colorectal adenocarcinoma cells was investigated by proliferation tests.

Patients and Methods

Ethics Statement

The study was approved by The Ethics Committee of College of Basic Medical Sciences and the First Hospital of Jilin University, China, and written informed consents were obtained from all participants prior to this study.

Materials

Serum and cancer tissues of 49 patients with colorectal adenocarcinoma were obtained from the tissue bank of the First Hospital of Jilin University. The patients had no obvious family history of other carcinomas. Their preoperative serum CEA levels were recorded. Post-operative records were reviewed to collect their pathological information. All patients were staged according to Dukes' staging criteria which are widely accepted and commonly applied in the clinical practice.

Twenty-four patients with benign colorectal polyps were enrolled. The diagnosis of colorectal adenocarcinoma in this cohort was ruled out by detailed colonoscopic investigation. These patients also had no history of hepatic or renal disorders and trauma. Blood samples of patients from this group were collected and centrifuged within 24 h of venepuncture, then stored at -80°C.

In addition, 20 healthy subjects that attended for regular check-ups were recruited as a negative control.

Furthermore, the sera of 8 postoperative patients with colorectal adenocarcinomas were assayed to detect the level of activin A as compared to their preoperative state. These blood samples were obtained at least 4 days after their surgeries to avoid the elevation of activin A in response to surgical trauma.

Enzyme-Linked Immunosorbent Assay for Activin A

Serum activin levels were assayed in patients with colorectal adenocarcinomas, benign polyps and in healthy subjects in triplicate with the use of ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Serum level of CEA in benign polyps and carcinoma patients were also assayed by ELISA.

Immunohistochemical Staining for Activin A

Tissue samples were obtained from the Endoscopy Center of the First Hospital of Jilin University. Colorectal adenocarcinoma tissues and benign polyps were collected and fixed in 4% paraformaldehyde for 24 h at room temperature. 3% hydrogen peroxide was used to block endogenous peroxidase for 30 min at RT. Nonspecific reactivity was blocked by pre-incubating the sections in 2% bovine serum albumin (BSA) in PBS for 30 min. Then the sections were incubated with polyclonal rabbit anti-activin antibody (1:300) overnight, and processed with biotinylated secondary antibodies for 10 min at RT followed by incubation with streptavidin-horseradish peroxidase for 10 min. Immunoreactive products were visualized in 0.03% H_2O_2 and 0.05% diaminobenzidine (DAB). The sections were dehydrated, cleared, mounted and observed under inverted microscope, and photographs were taken.

RT-PCR

Total RNA was extracted from two benign polyps and six colorectal carcinoma tissue samples using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, UK). 1 μg RNA samples were then reverse-transcribed with specific antisense primers by using the one-step RT-PCR kit in accordance with the manufacturer's instructions (Takara Biotechnology Co, Otsu, Shiga Japan). The primers were designed as follows: activin A forward primer 5'-GAGAGGAGTGAACTGTTGCT-3' and reverse primer 5'-ATGACTGTTGAGTGGAAGGA-3', β-actin forward primer 5'-GACTTCAACAGCAACTCCCACTC-3' and reverse primer 5'-TAGCCGTATTCATTGTCATACCAG-3'. Thirty-five cycles of PCR were performed, with each cycle consisting of 30 s at 94°C, 30 s at 55°C and 50 s at 72°C, followed by a final 10 min extension

step at 72°C. PCR products were analyzed by 1.5% agarose gel electrophoresis, and stained with ethidium bromide for detection. The specific bands were analyzed using ImageMaster-VDS system (Pharmacia Biotech Company, Uppsala, Sweden).

Western Blotting

Electrophoresis of proteins (50 μg/lane) extracted from colorectal carcinoma tissues, adjacent tissues and benign polyps was carried out in denaturing 15% SDS polyacrylamide gels for 4 h at 70 V. After separation, the proteins were transferred onto nitrocellulose membranes for 2 h at 270 mA. After blocking by 3% non-fat dry milk, membranes were incubated overnight at 4°C with the appropriate antibodies. After extensive washings, membranes were incubated with goat anti-mouse or anti-rabbit IgG peroxidase-conjugated antibodies. The immune complexes were visualized by Epoch ECL electrochemical analyzer.

Statistical Analysis

The assayed serum results from three groups (healthy controls, benign polyps and CRC) were compared using one-way ANOVA. $p < 0.05$ was considered to be statistically significant. Sensitivity for colorectal adenocarcinoma was expressed as 100 x [the number of colorectal adenocarcinoma patients with positive activin A or tumor markers (true positive) / total number of colorectal adenocarcinoma patients (true positive + false negative)] %. Specificity for colorectal adenocarcinoma was expressed as 100 x [the number of colorectal benign subjects with negative activin A or tumor marker (true negative) / total number of colorectal benign subjects (true negative + false positive)] %. Data were expressed as means ± SD.

Results

Clinical Data of Patients

Demographic and clinical characteristics of the study participants are provided in Table I. There was no statistically significant difference ($p > 0.05$) in participants' age between the groups.

Serum Activin A Levels

Serum activin A level in patients with colorectal adenocarcinomas was significantly higher than that in patients with benign polyps and healthy control group ($p < 0.01$, Figure 1A). In contrast, the serum activin A levels were not significantly different between patients with benign polyps and healthy control group ($p > 0.1$). The sensitivity of serum activin levels was 75.5% (37/49) in patients with colorectal adenocarcinomas by 95% confidence interval in the healthy subjects as the cut-off value. When the sensitivity and specificity of activin A in patients with colorectal adenocarcinomas was plotted on a ROC curve (Figure 1B), the area under the curve was 0.705. These data indicated that serum activin A levels might be a useful biomarker for diagnosis of colorectal adenocarcinoma.

Table I. Demographic characteristics and clinical data for healthy subjects and patients with benign polyps and colorectal adenocarcinomas.

	Healthy subjects	Benign polyps	Colorectal adenocarcinoma
Age (years)			
Mean±SD	55.8 ± 7.4	58.8 ± 7.6	61.7 ± 12.9
Range	42-69	40-70	24-82
Sex (n)			
Female	11	9	30
Male	9	15	19
Pathologic stage (n)			
I			10
II			13
III			26
Differentiation (n)			
Well			6
Moderately			34
Poorly			9
Total (n)	20	24	49

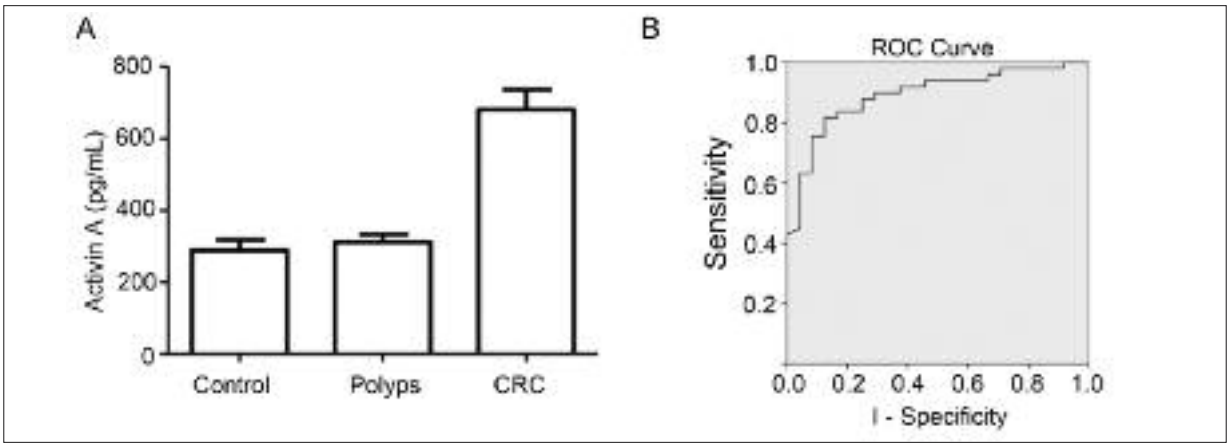

Figure 1. The levels of serum activin A in healthy subjects, subjects with benign polyps and colorectal adenocarcinoma patients. ***A,*** Serum activin A levels measured by ELISA. ACT – activin A. ***B,*** Receiver-operating characteristic (ROC) curve of activin A diagnosing colorectal adenocarcinoma. The area under the curve is 0.891.

Comparison of sensitivity and specificity between activin A and tumor marker CEA were conducted as shown in Table II. There was no significant difference in sensitivity between activin A and CEA (75.5% and 80%, respectively). In contrast, the specificity of activin A was significantly higher than that of CEA in the diagnosis of colorectal adenocarcinoma.

Comparison of Activin A Levels in PreoperAtive and Postoperative Patients with Colorectal Adenocarcinoma

Significantly lower serum activin A level was observed in postoperative patients compared to preoperative patients (Figure 2) indicating that elevated serum activin A level in preoperative patients might be correlated with the presence of tumor.

Relationship Between the Serum Activin Levels and the Stage and Pathological Grade of Colorectal Adenocarcinoma

Significant differences were observed within carcinoma group between the patients with Stage I of disease and Stages II and III. There was no significant difference between Stages II and III (Figure 3). Non-significant differences in serum activin A level were observed between patients with poorly differentiated colorectal adenocarcinoma and patients with moderately differentiated tumor. In addition, the differences in serum activin A levels between patients with and without lymph node infiltration were not significant. Well-differentiated carcinomas were not included in the analysis due to small sample size.

Table II. Comparison of sensitivity and specificity between the serum level of activin A and the level of CEA in colorectal carcinoma patients.

	Sensitivity	Specificity
Activin A	75.5%	85%
CEA	80%	63%

Expression of Activin A in Colorectal Adenocarcinoma Tissues

RT-PCR

PCR analysis indicated that colorectal adenocarcinomas had higher βA-subunit mRNA ex-

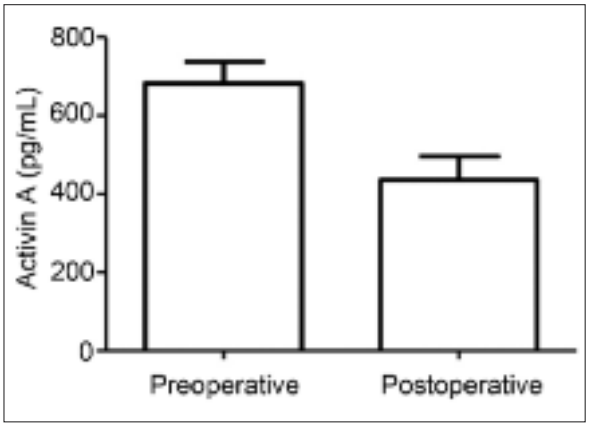

Figure 2. Serum level of activin A in postoperative and preoperative patients.

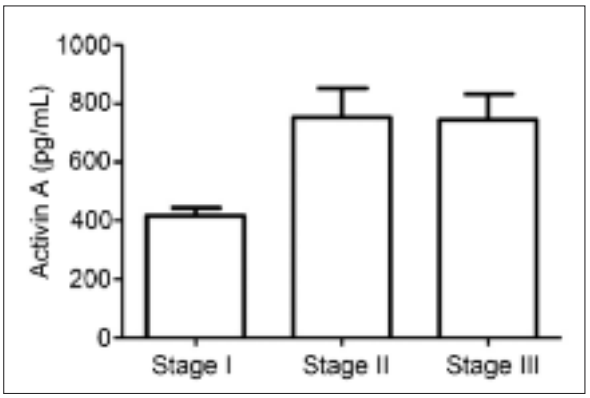

Figure 3. Serum levels of activin A at different stages of colorectal carcinomas.

pression level compared to benign polyps, as shown in Figure 4. The βA-subunit mRNA expression level in 4 out of 6 analyzed colorectal cancer samples was high. The level of β-actin expression in all analyzed samples was identical (data not shown). These findings are consistent with the data on the serum level of activin A.

Immunohistochemical Staining

Immunohistochemical staining was performed to examine the expression of activin A in colorectal adenocarcinoma. The results showed that immunoreactivity could be observed in colorectal adenocarcinoma tissues (Figure 5A-D), as compared to negative controls (Figure 5E, F). On the contrary, benign polyps merely presented with a low level of expression in interstitial tissues while there was no staining in parenchymal tissues (Figure 5G, H).

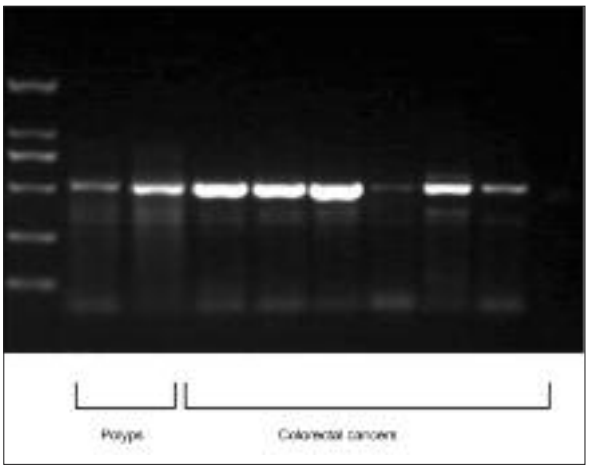

Figure 4. Expression level of βA-subunit mRNA in colorectal cancer and benign polyp samples analyzed by RT-PCR.

Western Blotting

Western blotting analysis further confirmed the elevated expression level of activin A, as shown in Figure 6. The carcinoma samples exhibited significantly higher expression level of activin A compared to the adjacent tissues and benign polyp.

Discussion

Colorectal cancer (CRC) incidence has been rising strongly in the industrialized nations. This growth correlates well with the economic development of countries[10], making colorectal cancer the second largest cause of cancer-related mortality in the developed world[11]. Population-based screening programs can reduce CRC-related mortality through early detection and treatment of the disease, as well as removing of premalignant lesions (adenoma)[12]. Currently, the early diagnostics of CRC is mainly based on colonoscopic findings and measurement of CEA level. Even though conventional colonoscopy is considered to be the ideal technique for the CRC screening programs, the participation level in such programs is usually low, and colonoscopic findings remain operator-dependent to some extent. Carcinoembryonic antigen (CEA) is a complementary index and is commonly used in CRC screening. It is, however, insufficient for early detection of the disease due to the limited sensitivity and specificity[3]. In addition, imaging tests such as CT scan, MRI and endoscopic ultrasound investigations are applied to stage the disease, but their roles are still controversial[13]. The development of new approaches is required to improve the screening and diagnostic effectiveness.

There is increasing evidence that activins, the members of the transforming growth factor β (TGF-β) superfamily, could play important roles in carcinogenesis[14]. Wildi et al[9] reported that activin A is overexpressed in human colorectal tumors. However, its exact role has not yet been fully investigated. In present study, we measured the levels of activin in the sera of patients diagnosed with CRC and colorectal polyps. The higher serum activin A levels in patients with CRC compared to patients with benign polyps were confirmed by our assays. However, activin A can also be elevated by inflammations and other factors. To identify the sources of elevated activin levels in CRC patients, immunohistochemical

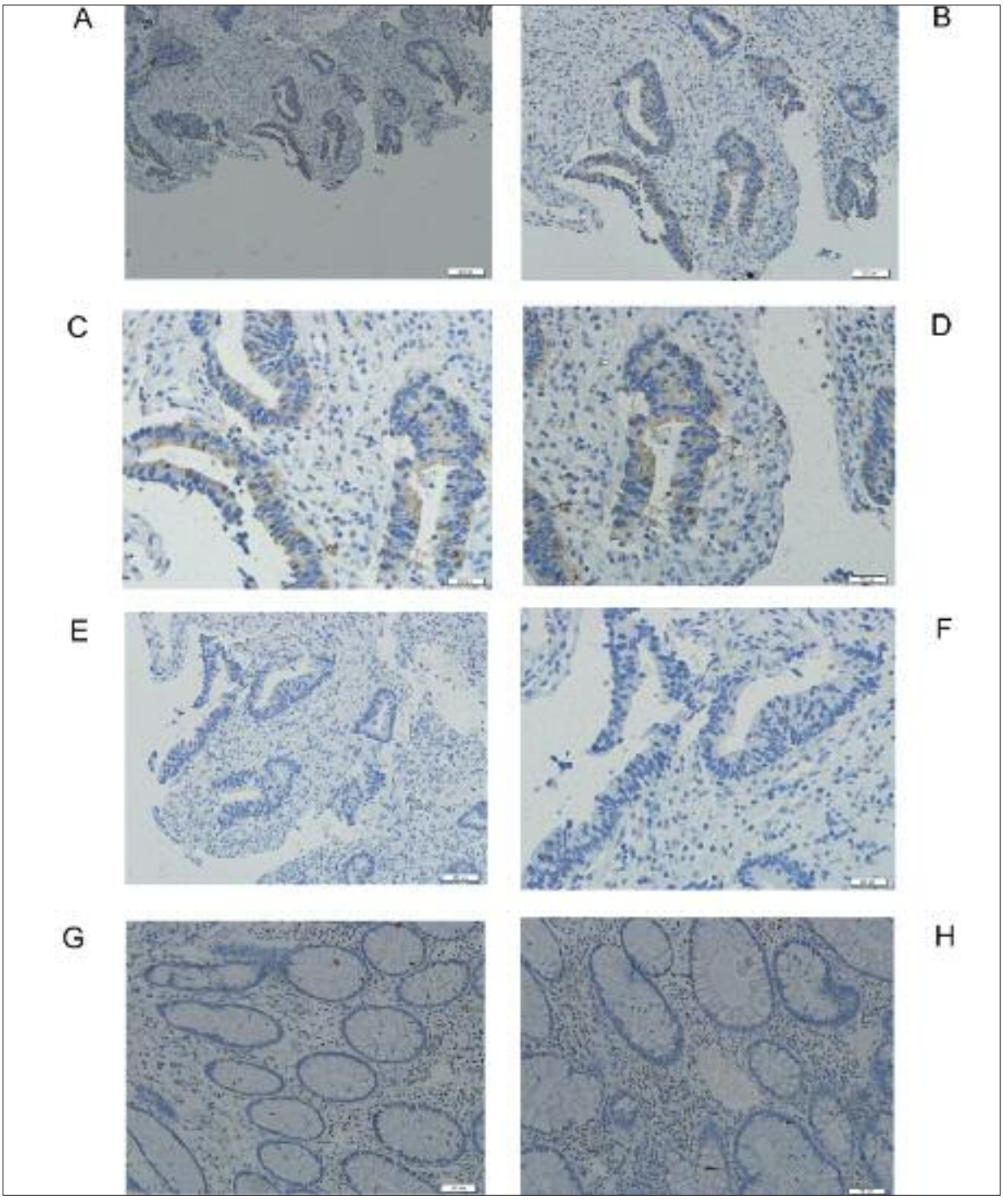

Figure 5. Immunohistochemical staining of colorectal adenocarcinoma tissues ***(A-D)***, negative controls samples ***(E, F)***, and benign polyps samples ***(G, H)***.

staining and RT-PCR were performed. On the immunohistochemical staining, activin expression was more pronounced in colorectal adenocarcinoma cells and parenchymal tissues rather than interstitial tissues. This suggests that increased levels of activin in the peripheral blood of colorectal adenocarcinoma patients might be directly linked to the presence of tumor secreting

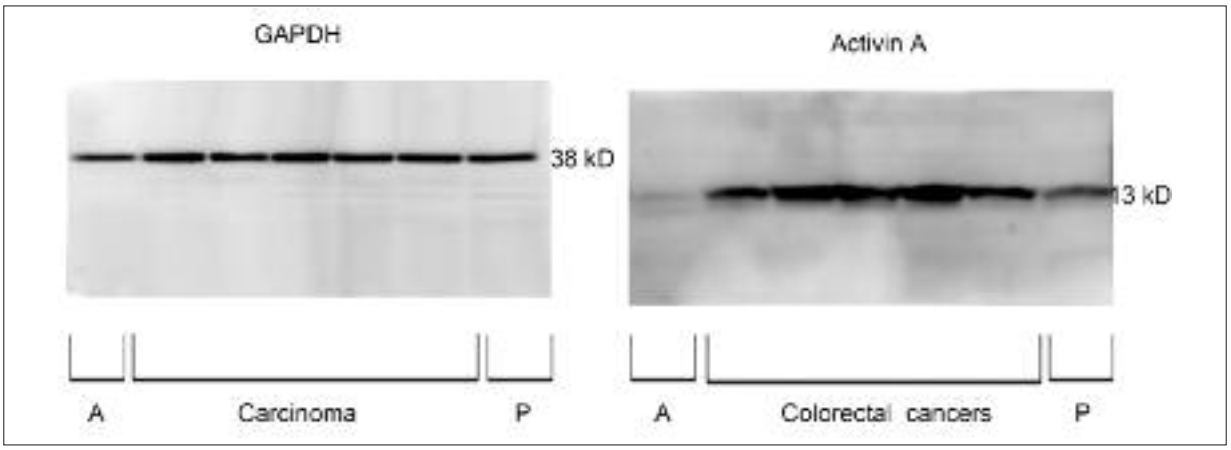

Figure 6. Expression of activin A analyzed by Western blotting. A: Adjacent tissues. P: Benign polyp.

activin into the blood stream. RT-PCR further demonstrated that colorectal adenocarcinomas express higher level of βA-subunit mRNA compared to benign polyps. (It should be noted, however, that the elevated expression of βA-subunit was seen not in all analyzed samples – this fact is likely to reflect the heterogeneity of clinical samples and suggest that the role of activin A might be different in different subtypes of colorectal adenocarcinoma.) Moreover, the lower postoperative serum activin A level further indicated that the source of serum activin A is likely to be the carcinoma tissues. Taken together, these evidences strongly suggest that activin A is directly secreted by colorectal adenocarcinoma cells. The result of Western blotting further confirmed the expression of activin A in colorectal adenocarcinoma tissue samples. In comparison with benign polyps and adjacent tissues, the overexpression of activin A in colorectal adenocarcinoma suggests that it might play a role in the carcinogenesis and further development of tumor.

CEA is a widely recognized and accepted tumor marker for diagnosis of colorectal cancers in clinical practice[15]. In this study, we performed a comparison between CEA and activin A. Activin A had similar sensitivity to CEA. Interestingly, activin A has significant higher specificity compared to CEA indicating that measuring the activin A level in serum might be used as a complementary assay for correction in cases of false-positive CRC diagnoses predicted by CEA. The data presented in this article suggest that activin might be a useful predictor for the diagnosis of colorectal adenocarcinoma with high sensitivity and specificity.

Conclusions

Our results demonstrate that elevation of activin A level in the serum of patients with colorectal adenocarcinoma is caused by secretion of activin A by adenocarcinoma cells. This process might function as a promoting factor in the development of adenocarcinoma. Thus, activin A may be used as a potential biomarker for diagnosis of colorectal adenocarcinoma in humans and a novel target in therapeutic approaches targeting colorectal cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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