Activin A promotes cell proliferation, invasion and migration and predicts poor prognosis in patients with colorectal cancer

NOBUYA DAITOKU[1], YUJI MIYAMOTO[1], YUKIHARU HIYOSHI[1], RYUMA TOKUNAGA[1], YUKI SAKAMOTO[1], HIROSHI SAWAYAMA[1], TAKATSUGU ISHIMOTO[1,2], YOSHIFUMI BABA[1], NAOYA YOSHIDA[1] and HIDEO BABA[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556; [2]Gastrointestinal Cancer Biology, International Research Center for Medical Sciences, Kumamoto University, Kumamoto 860-0811, Japan

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Abstract. Activin A is a member of the transforming growth factor-β superfamily of cytokines and displays various pathophysiological activities, including regulation of muscle catabolism and atrophy. Activin A expression is upregulated in several human cancer types and in certain pathologies, its expression is associated with poor prognosis. In the present study, activin A expression was assessed in colorectal cancer (CRC) tissue specimens from 157 patients with primary CRC and the relationship between activin A levels and clinicopathological characteristics, including skeletal muscle mass, and prognosis, was determined. Furthermore, the effects of knockdown of endogenous or exposure to exogenous activin A on the malignant behavior of human CRC cell lines were investigated *in vitro*. The results indicated that activin A mRNA was significantly upregulated in CRC tumor tissues compared with normal intestinal epithelium. High activin A expression was significantly associated with shorter cancer-specific survival (P=0.047) and overall survival (P=0.014). According to a multivariate analysis, tumor activin A levels were an independent prognostic factor for overall survival (P=0.001). However, activin A mRNA levels were not associated with the skeletal muscle index. The *in vitro* experiments demonstrated that exposure to exogenous activin A increased the proliferation, invasion and migration of CRC cell lines, whereas knockdown of endogenous activin A had the opposite effects. In conclusion, activin A is an autocrine and paracrine regulator of CRC cell proliferation and high tumor expression of activin A is associated with poor prognosis in patients with CRC.

Correspondence to: Professor Hideo Baba, Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
E-mail: hdobaba@kumamoto-u.ac.jp

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Introduction

Colorectal cancer (CRC) is currently the third most common cause of cancer-related mortality in the economically developed world and is on track to increase in ranking in the coming decades (1). Surgical resection in combination with systemic chemotherapy offers the only hope of cure or long-term survival for patients with CRC. However, the disease recurs in ~30% of patients and better treatment options are required to improve prognosis (2). Only a small number of specific diagnostic or therapeutic tools are currently available, to a large part due to the currently limited understanding of the molecular pathogenesis of the disease. Although certain molecular targeted therapies have proven efficacious in CRC (3-7), there is an urgent requirement to identify novel therapeutic targets. Furthermore, as CRC has a high relapse rate even early after radical resection, there is a requirement to identify additional biomarkers that may complement those currently available to predict early postoperative recurrence and poor prognosis for patients with CRC (3,5,6,8).

Previous studies by our group reported that sarcopenia is an independent unfavorable prognostic factor after curative resection in patients with CRC (9,10). Sarcopenia, defined as a decrease in muscle mass associated with aging and disease, is common in cancer patients (11), and other studies also indicated that it is a poor prognostic factor for various types of cancer (10,12-14). Sarcopenia is associated with decreased survival in patients with CRC undergoing curative resection (9), suggesting that understanding the molecular events underlying skeletal muscle degradation may identify potential novel therapeutic targets for these patients.

One molecule associated with the regulation of skeletal muscle mass is activin A, a member of the transforming growth factor-β (TGF-β) family of proteins. In addition to promoting skeletal muscle degradation and atrophy, activin A displays an array of biological activities (15). Under physiological conditions, activin A exerts its effects on cells by binding to type II receptors, which induces its dimerization with type I receptors. Once engaged, the activated type I receptor complex, which has serine/threonine kinase activity, phosphorylates SMAD2/3 and recruits SMAD4, the main signal transducers of the TGF-β family receptors. The SMAD complex then

translocates to the nucleus where it promotes transcription of a panel of genes involved in the regulation of cell development and proliferation, including muscle catabolism (16).

Activin A is expressed and secreted by a number of human cancer cell lines (17) and its overexpression has been associated with poor prognosis in various malignant tumor types, including esophageal adenocarcinoma, lung cancer and gastric cancer (18-22). In the present study, activin A expression was determined in CRC tissues and its association with skeletal muscle mass and its prognostic significance were examined. In addition, the mechanisms underlying the involvement of activin A in CRC were explored *in vitro*.

Materials and methods

Patients and tissue samples. The study population consisted of 157 patients with CRC who underwent surgical resection at the Department of Gastroenterological Surgery, Kumamoto University Hospital (Kumamoto, Japan) between January 2008 and December 2012. The mean observation period for the cohort was 57 months (range, 1-91 months). The clinical characteristics of the 157 patients are summarized in Table I. This study included 83 males and 74 females ranging in age from 34 to 86 years. The patients underwent imaging examination, such as colonoscopy and enhanced computed tomography, for CRC diagnosis and staging prior to surgery. The diagnosis was pathologically confirmed using biopsy specimens. Patients who had received preoperative chemotherapy or emergency surgery were excluded. CRC tissue or paired normal epithelial tissue was obtained at the time of surgical resection, snap-frozen and stored at -80˚C until use. The present retrospective, non-interventional, observational study was approved by the institutional ethics committee of Kumamoto University Hospital (14 June 2019/approval no. 1047) and performed in accordance with the Declaration of Helsinki from 1975.

Validation analysis in The Cancer Genome Atlas (TCGA) database. To validate the association between activin A expression and prognosis for patients with CRC, the activin expression data and related clinical information of patients with CRC were obtained from the TCGA database (http://www.cbioportal.org). The patients with CRC in the TCGA dataset were divided into two groups according to the median activin A expression. The cumulative overall survival (OS) rate of the patients was determined using Kaplan-Meier survival analysis with a log-rank test.

Cell lines and cell culture. The human CRC cell lines LoVo and SW480 were purchased from RIKEN Bioresource Center Cell Bank and the Japanese Collection of Research Bioresource Cell Bank, respectively. LoVo and SW480 cells were cultured in Ham's and RPMI media (both from Wako Pure Chemical Industries, Ltd.), respectively, supplemented with 10% fetal bovine serum (Mediatech, Inc.). Cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2 and were confirmed to be negative for mycoplasma infection prior to use.

Measurement of skeletal muscle area. The skeletal muscle area was retrospectively measured on preoperative computed tomography scans at the third lumbar vertebra (L3) level in the inferior direction with the patient in the supine position. In brief, a three-dimensional image analysis system (Volume Analyzer SYNAPSE VINCENT; Fujifilm Medical) was used to measure pixels using a window width of -30 to 150 HU to delineate the muscle compartments and compute the cross-sectional area of each in centimeters squared (cm^2). The cross-sectional area of the muscle (cm^2) at the L3 level computed from each image was normalized by the square of the height (m^2) to obtain the skeletal muscle index (SMI) expressed in cm^2/m^2 (9,10).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from frozen tissue samples or CRC cell lines using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and the concentration of purified RNA was measured by comparing absorbance at 260 nm (A260) and A280 using a Nanodrop® 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was generated from total RNA using a ReverTra Ace qPCR RT kit (Toyobo Life Science) according to the manufacturer's protocol and was subsequently used as a PCR template. Real-time q-PCR was performed as described previously (21). mRNA levels were measured in technical duplicates and the relative level of activin A mRNA was calculated as the fold change relative to β-actin (ACTB) mRNA. The samples were quantified using the $2^{-\Delta\Delta Cq}$ method (23). The primers used were as follows: Activin A (INHBA) forward, 5'-CCTCGGAGATCATCACGTTT-3' and reverse, 5'-CCC TTTAAGCCCACTTCCTC-3'; and ACTB forward, 5'-ATT GGCAATGAGCGGTTC-3' and reverse, 5'-CGTGGATGC CACAGGACT-3'.

Immunohistochemical (IHC) staining. CRC and normal epithelial samples were formalin-fixed and paraffin-embedded. Blocks were cut into 3-μm sections, which were deparaffinized and rehydrated. Activin A antigen was retrieved by autoclaving in a pH 9 buffer solution for 15 min. Subsequently, the sections were incubated overnight at 4˚C with goat anti-activin A antibody (1:100 dilution; cat. no. A1594; MilliporeSigma). The sections were washed in PBS and incubated with horseradish peroxidase-conjugated mouse anti-goat secondary antibody (1:50 dilution; cat. no. K8000; EnVision goat; Dako; Agilent Technologies, Inc.) at room temperature for 30 min. Color development was achieved by the addition of 3,3'-diaminobenzidine [Wako Tablet; cat. no. 040-27001 (5 mg); Dako; Agilent Technologies, Inc.] followed by counterstaining with hematoxylin.

Cell transfection. A total of two activin A-specific small interfering RNAs (siRNAs; siActivin A #1 and #2; Silencer Select s7434 and s7436; Thermo Fisher Scientific, Inc.) and a negative control siRNA (siCtrl, Stealth RNAi; Invitrogen; Thermo Fisher Scientific, Inc.) were employed. Pilot experiments were performed to determine the optimal siRNA concentration (10 μM) for inhibition of activin A expression to <30% of the levels in siCtrl-transfected cells. Cells were seeded in 6-well plates at a density of 10^5 cells/well in 2.5 ml medium and incubated for 24 h. The cells were then transfected with 10 μM siActivin A or siCtrl using Lipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's

Table I. Characteristics of the patients (n=157).

Factor	Value
Age, years	64 (34-86)
Sex	
Male	83
Female	74
Body mass index, kg/m^2	22.6 (15.7-34.2)
SMI, cm^2/m^2	47.0 (28.4-85.0)
Location	
Colon	113
Rectum	44
Tumor stage	
T1	24
T2	28
T3	73
T4	32
Lymph node metastasis	
Present	59
Absent	98
Lymphatic invasion	
Present	51
Absent	106
Venous invasion	
Present	67
Absent	90
pStage	
I	33
II	49
III	39
IV	36
CEA, ng/ml	24.7 (0.6-18021)
CA19-9, U/ml	39.7 (0.6-34708)

Values are expressed as median (range) or n. SMI, skeletal muscle index; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

protocol. After 48 h of transfection, the supernatant was removed, the cells were washed with PBS and the experiments were performed.

Cell proliferation assay. Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. LoVo and SW480 cells were seeded in 96-well plates at a density of 3.0x10^3 cells/well in 100 μl medium and incubated overnight at 37˚C. Aliquots of 10 μl/well CCK-8 solution were then added to the cells after 0, 24, 48, 72 or 96 h of incubation and the plates were incubated for an additional 90 min. The absorbance at 450 nm was then read using a microplate reader (SPECTRAmax PLUS 384 microplate spectrophotometer; Scientific Equipment Source). Each experiment was performed in triplicate.

Invasion assay. Cell invasion was measured assay using BioCoat Matrigel invasion chambers (24-well plates, 8-μm pore size; BD Biosciences) according to the manufacturer's protocol. In brief, LoVo and SW480 cells were resuspended in medium at a concentration of 10^5 cells/ml and 500 μl of the cell suspension was placed in the upper chambers. The same medium supplemented with 10% fetal bovine serum (FBS; Mediatech) was placed in the lower chamber and the plates were incubated for 22 h. The cells on the upper surface of the membrane were then removed using a cotton swab and the cells on the lower surface were fixed with 100% ice-cold methanol for 2 min, followed by staining with toluidine blue for 2 min at room temperature. The membrane was rinsed with water and examined using a microscope (MRP-3001; R&D Systems, Inc.). The number of invaded cells was counted in five microscopic fields per membrane (magnification, x40).

Migration assay. Migration was measured using 6-well plates coated with 200 μl/well Matrigel® (BD Biosciences). LoVo and SW480 cells were resuspended at a concentration of 4.0x10^4 cells/ml in each medium, plated at 200 μl/well and allowed to adhere for 12 h. The plates were then imaged with a KEYENCE BZ-X700 all-in-one fluorescence microscope equipped with a CO_2 and temperature-controlled chamber and time-lapse tracking system (Keyence Corporation). Phase contrast images were acquired every 10 min for 24 h and converted to video files using a BZ-X Analyzer (Keyence Corporation). Cell migration was analyzed using video editing analysis software VW-H2MA (Keyence Corporation) and the tracking data were processed using Excel 2010 (Microsoft Corporation) to generate xy coordinate plots and allow measurement of the distance moved. Migration distance was calculated by randomly selecting three cells in each well, tracking their movement for 15 sec and plotting the average value (n=3) of the distances moved on a graph.

Statistical analysis. Continuous variables are expressed as the median and range. Continuous and categorical variables were compared using the Mann-Whitney U-test and χ^2 test, respectively. Survival analyses were performed using the Kaplan-Meier method with the log-rank test. The correlation between activin A mRNA levels and SMI was assessed by calculating Spearman's rank correlation coefficient ρ. OS was calculated as the duration from the date of surgery until death or the last follow-up. Cancer-specific survival (CSS) was calculated from the time of diagnosis to the time of death from any cancer or last follow-up. Variables with significance at P<0.05 in the univariate analysis were included in multivariate analysis using stepwise backward elimination procedures. The Cox proportional hazards model for multivariate analysis was used. All statistical analyses were performed using JMP version 13.1 (SAS Institute, Inc.). All P-values were two-sided and P<0.05 was considered to indicate statistical significance. The term 'prognostic marker' is used according to the REMARK guidelines (24).

Results

Associations between activin A expression in CRC tissues and clinicopathological characteristics. To determine whether the

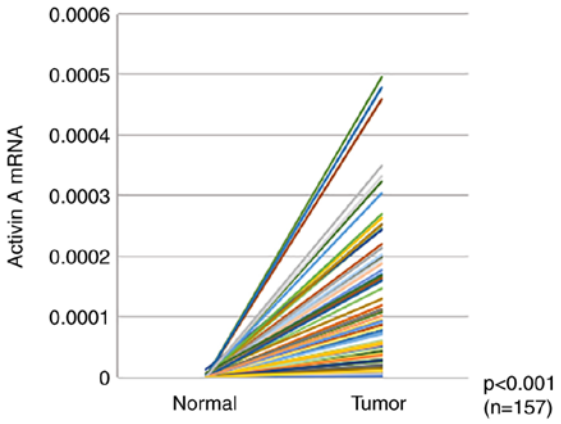

Figure 1. Activin A mRNA expression levels in colorectal cancer tissues and matched normal intestinal epithelia tissue (n=157).

expression level of activin A is elevated in CRC, RT-qPCR analysis of 157 matched pairs of CRC and normal epithelial tissue samples was performed. Activin A mRNA expression was significantly higher in CRC tissues than in normal epithelia (P<0.001; Fig. 1). To assess the associations between activin A mRNA levels and clinicopathological factors, patients were assigned to high (n=78) and low (n=79) activin A expression groups using the median value as the cut-off. However, none of the clinicopathological factors examined, including tumor location and metastasis/invasion status, was significantly associated with activin A mRNA levels in tumor tissues (Table II).

Correlation between tumor expression of activin A and SMI. Next, the association between activin A mRNA expression and the SMI was assessed using Spearman's rank correlation analysis. As presented in Fig. 2, there were no significant correlations between activin A mRNA expression in CRC tissues and the SMI for the full patient cohort (n=157, ρ=0.037, P=0.651), males (n=93, ρ=0.083, P=0.938) or females (n=64, ρ=0.189, P=0.141). Thus, the elevated expression of activin A in CRC tumors appeared to be unrelated to the SMI.

Association between activin A expression and patient survival. Kaplan-Meier curves were generated to assess the OS and CSS of patients with CRC according to activin A tumor expression levels (Fig. 3). It was indicated that patients with high tumor expression of activin A had significantly poorer OS (P=0.014) and CSS (P=0.047, log-rank test) than patients with low tumor expression. Next, factors associated with poor OS were evaluated by univariate and multivariate Cox regression analyses. Univariate analysis revealed that an age of ≥75 years, tumor stage, lymph node metastasis, CA19-9 level >37 U/l and high activin A expression were significantly associated with poor OS (Table III) and multivariate analysis demonstrated that an age of ≥75 years [hazard ratio (HR)=4.678, P=0.009], lymph node metastasis (HR=3.372, P=0.009), CA19-9 level >37 (HR=3.591, P=0.015) and high activin A expression (HR=4.287, P=0.001) were independent risk factors for poor OS (Table III). To validate the present results, the association of activin A mRNA levels with survival of 443 patients with CRC was determined using a dataset from the TCGA database. Kaplan-Meier analysis confirmed that high activin A expression (n=333) was significantly associated with poor OS (P=0.039; Fig. S1).

Table II. Patients' characteristics and clinicopathological factors in patients with colorectal cancer according to activin A expression.

	Activin A mRNA expression		
Factor	Low (n=78)	High (n=79)	P-value
Age ≥75 years	25 (32)	30 (38)	0.585
Female sex	39 (50)	35 (44)	0.162
Body mass index ≥25.0 kg/m^2	19 (24)	19 (24)	0.933
Location in rectum	13 (17)	29 (37)	0.055
Tumor stage T3-T4	55 (71)	60 (76)	0.221
Lymph node metastasis	29 (37)	30 (38)	0.778
Lymphatic invasion	26 (33)	25 (32)	0.502
Venous invasion	30 (38)	37 (47)	0.425
CEA >3.4 ng/ml	24 (31)	28 (35)	0.440
CA19-9 >37.0 U/ml	12 (15)	15 (19)	0.590

Values are expressed as n (%). CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

Proliferation, invasion and migration of human CRC cell lines exposed to activin A in vitro. Next, *in vitro* experiments were performed to clarify the biological activities of activin A in CRC cells. The subcellular pattern of activin A expression in CRC cells was determined by IHC staining of sections of resected specimens. Activin A expression was not present in normal epithelium but in certain CRC tissues. Activin A staining was observed throughout the cytoplasm of CRC cells (Fig. S2). The present results thus indicated that high activin A expression is associated with unfavorable patient outcomes, but not with sarcopenia, as indicated by the SMI analysis. Therefore, it was next queried whether exposure to activin A directly affects the malignant behaviors of CRC cells *in vitro*. First, RT-qPCR analysis of a panel of human CRC cell lines was performed, which indicated that LoVo and SW480 cell lines expressed significantly higher levels of endogenous activin A mRNA than the other cell lines tested (Fig. S3). LoVo and SW480 cells were exposed to exogenous activin A at 10 ng/ml for up to 96 h and the effects on cell proliferation, migration and invasion were analyzed. Cells exposed to activin A displayed significantly increased proliferation (P<0.05; Fig. 4A), invasion (P<0.05; Fig. 4B) and migration (P<0.0; Fig. 4C) compared with untreated control cells. Similar results were obtained in SW620 cells, which had lower activin A expression than LoVo cells (Fig. S4). Thus, activin A may act on CRC cells to promote behaviors associated with malignancy.

Proliferation, invasion and migration of human CRC cell lines subjected to activin A knockdown. As LoVo and SW480

Table III. Univariate and multivariate analyses of factors influencing overall survival in colorectal cancer.

	Univariate analysis			Multivariate analysis		
Factor	HR	95%CI	P-value	HR	95%CI	P-value
Age ≥75 years	1.801	1.108-2.898	0.018	4.678	2.018-11.15	0.009
Female sex	1.185	0.733-1.902	0.485			
Body mass index ≥25.0 kg/m^2	0.564	0.271-1.052	0.073			
Location in rectum	0.933	0.552-1.529	0.788			
Tumor stage T3-T4	2.949	1.218-9.704	0.014	2.711	0.839-12.12	0.100
Lymph node metastasis	2.835	1.754-4.673	<0.001	3.372	1.344-9.028	0.009
CEA >3.4 ng/ml	1.005	0.624-1.614	0.982			
CA19-9 >37.0 U/ml	2.034	1.166-3.408	0.014	3.591	1.313-9.048	0.015
Activin A high	2.543	1.157-5.982	0.020	4.287	1.776-11.14	0.001

HR, hazard ratio; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

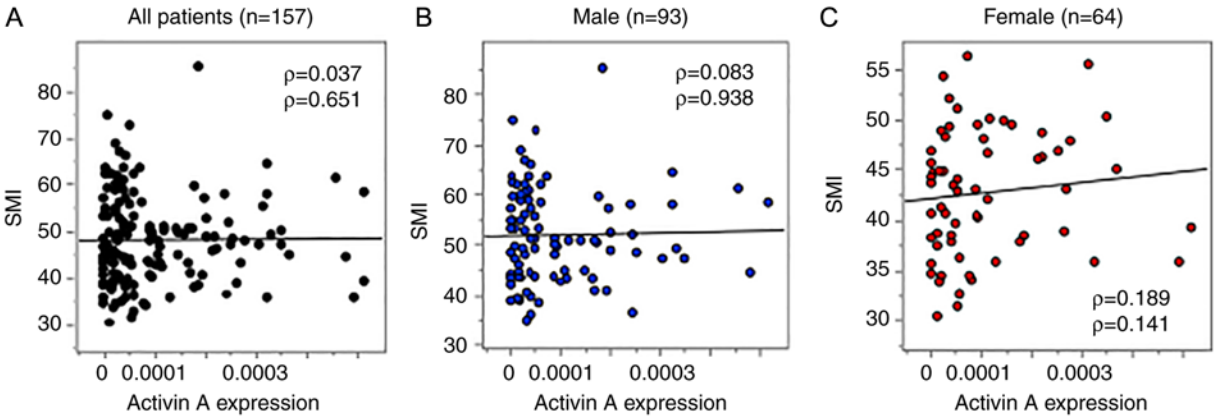

Figure 2. Spearman's rank correlation between tumor activin A expression and preoperative SMI. Correlation analyses between activin A mRNA levels determined by reverse transcription-quantitative PCR and SMI in (A) all patients (n=157), (B) males (n=93) and (C) females (n=64). ρ, Spearman's rank correlation coefficient; SMI, skeletal muscle index.

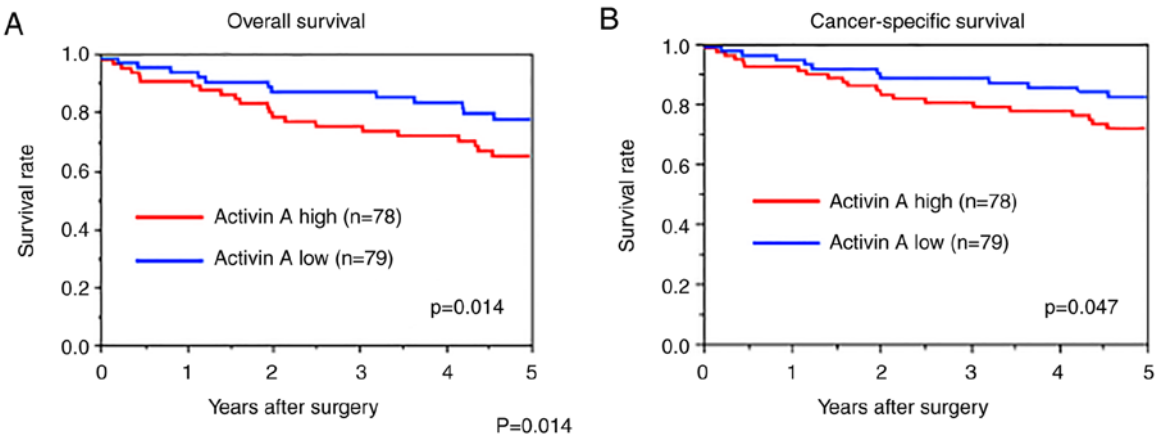

Figure 3. Kaplan-Meier survival analyses of patients with colorectal cancer (n=157) according to activin A mRNA expression in tumors. (A) Overall survival. (B) Cancer-specific survival. Patients were stratified using the median activin A expression level as the cut-off.

cells express high endogenous levels of activin A (Fig. S3), it was next investigated whether siRNA-mediated knockdown of activin A affected malignant cell phenotypes *in vitro*. Two independent activin A-targeting siRNAs (siActivin A #1 and #2) were evaluated and confirmed by RT-qPCR analysis to effectively suppress activin A mRNA expression to

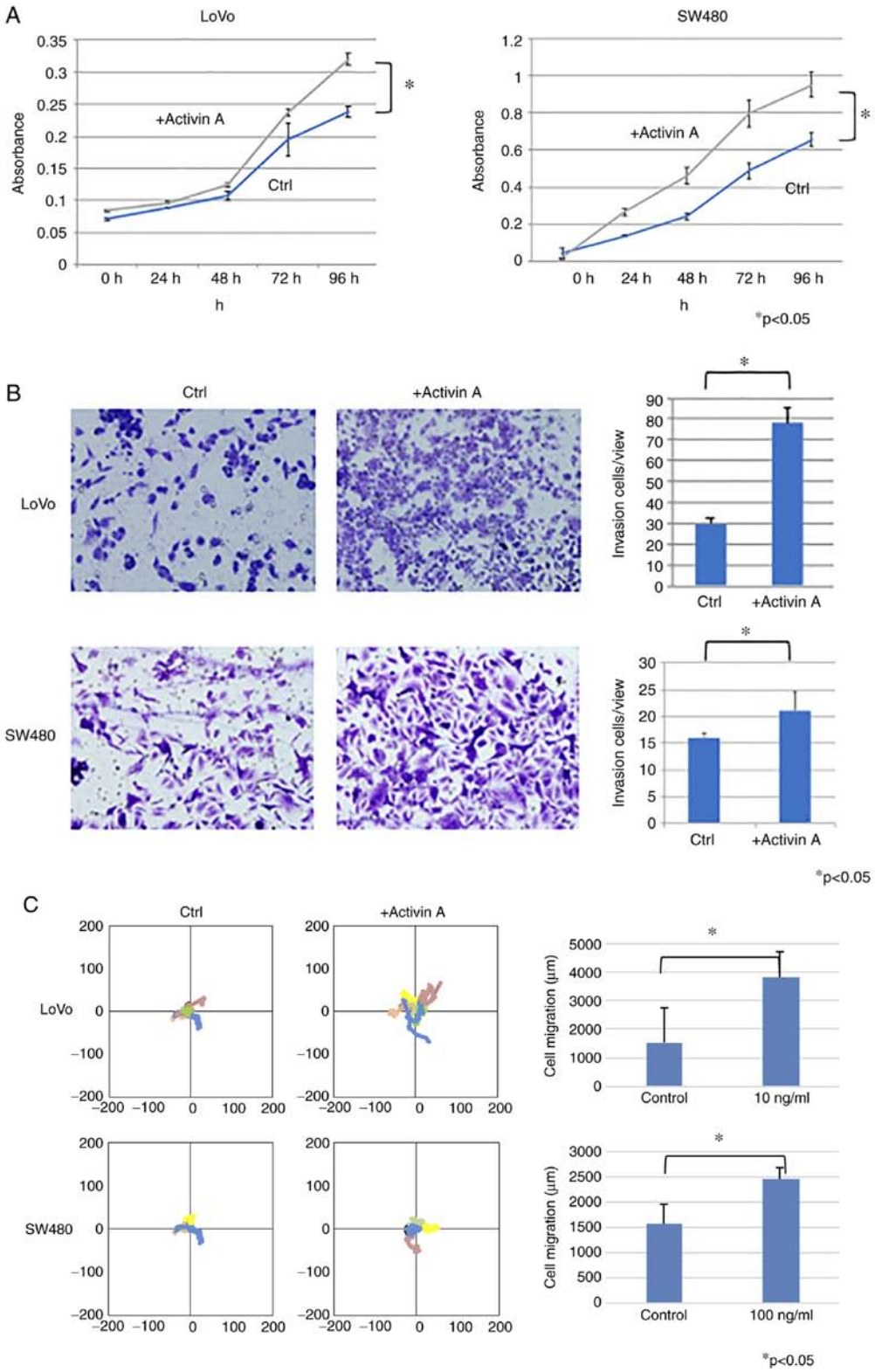

Figure 4. Proliferation, invasion and migration of colorectal cancer cell lines after exposure to activin A *in vitro*. (A) Cells were exposed to 10 ng/ml activin A or medium (Ctrl) for the indicated times and proliferation was measured using the Cell Counting Kit-8 assay. (B) Images (left) and quantification (right) of invasion of LoVo and SW480 cells after incubation with 10 ng/ml activin A or medium (Ctrl) in BioCoat Matrigel invasion chambers for 24 h (magnification, x40). (C) Images (left) and quantification (right) of migration of LoVo and SW480 cells exposed to 10 ng/ml activin A or medium (Ctrl) in Matrigel-coated six-well plates for 24 h. Values are expressed as the mean ± standard deviation of triplicates. *P<0.05. Ctrl, control.

levels <30% of those in LoVo or SW480 cells transfected with an siCtrl sequence (Fig. S5).

Compared with siCtrl-transfected cells, siActivin A #1- or #2-transfected LoVo and SW480 cells exhibited significantly decreased proliferation (P<0.05; Fig. 5A), invasion (P<0.05; Fig. 5B) and migration (P<0.05; Fig. 5C). These results suggested that endogenous activin A expression contributed to the malignant behavior of CRC cells.

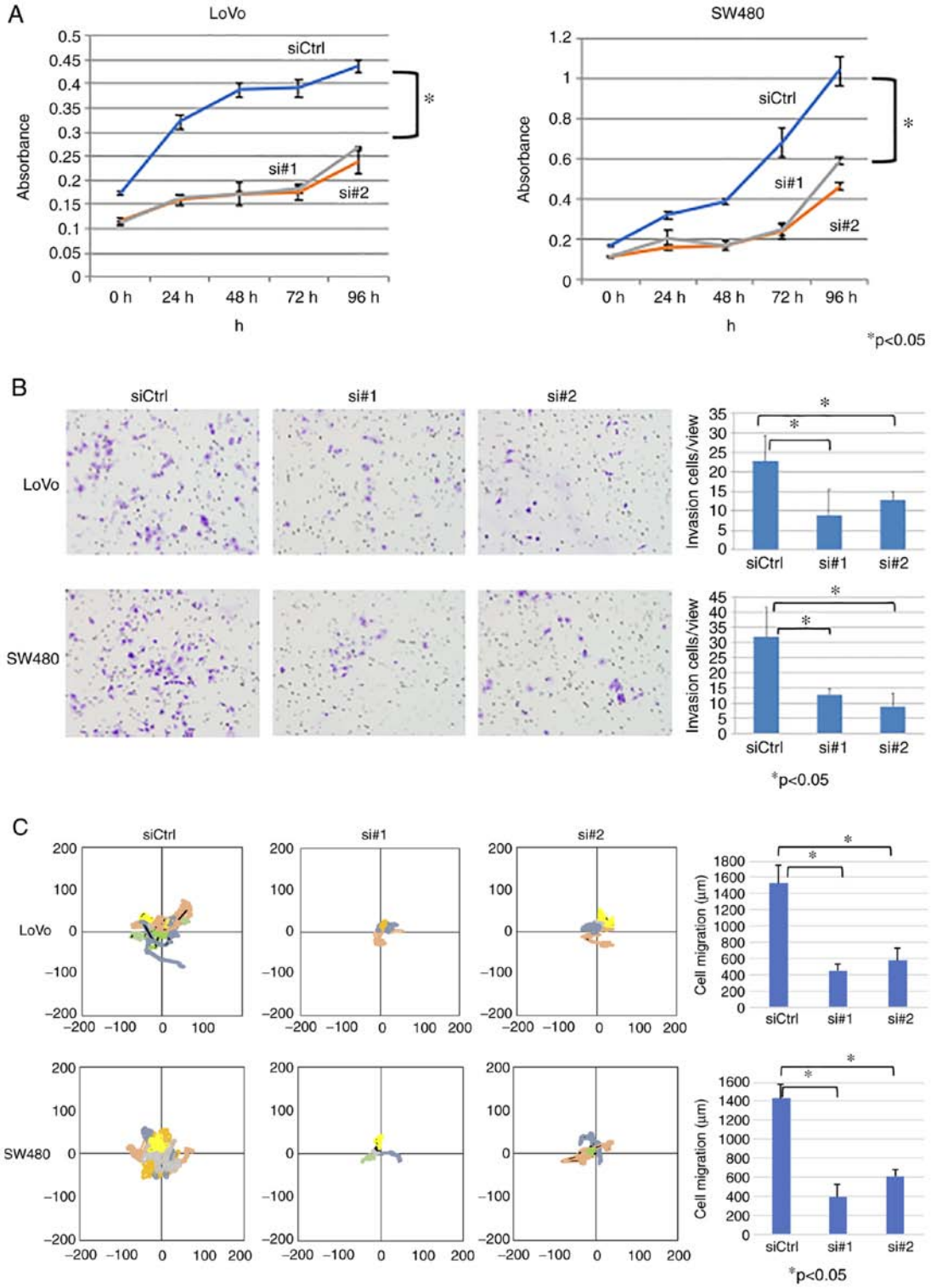

Figure 5. Proliferation, invasion and migration of colorectal cancer cell lines *in vitro* after activin A knockdown. Cells were transfected with one of two activin A-specific siRNAs (#1, #2) or a control siRNA (siCtrl) for 24 h and then incubated with activin A or medium. (A) Proliferation was measured using the Cell Counting Kit-8 assay at the indicated times. (B) Cells were plated in BioCoat Matrigel invasion chambers and invasion was assessed after 24 h (magnification, x40). (C) Cells were plated in Matrigel-coated wells and migration was assessed over 24 h. Values are expressed as the mean ± standard deviation of triplicates. *P<0.05. siRNA, small interfering RNA.

Discussion

Activin A has a number of important physiological roles, including induction of differentiation during vertebrate embryogenesis, neuronal differentiation and skeletal muscle cell degradation. Activin A circulates in the blood and is secreted by the gonads, pituitary gland and placenta. Previous studies reported that high tumor expression of activin A is associated with poor prognosis in patients with certain types of gastrointestinal cancer (18-21). Consistent with this, the present

study demonstrated that activin A was highly expressed in CRC tissues compared with matched normal intestinal epithelium and that high expression was an independent predictor of poor prognosis for patients with CRC.

In the early stages of epithelial cancers, TGF-β family cytokines function as tumor suppressors and inhibit cell proliferation. However, as the cancer progresses, TGF-β cytokines become tumor promoters and induce cancer metastasis by enhancing cell migration and invasion. Numerous studies have examined the involvement of activin A in cancer malignancy (22,25,26). For instance, Hoda *et al* (25) reported that activin A is involved in the malignant transformation of pleural mesothelioma through regulation of cyclin D. The results of the present study provide support for these earlier findings.

Antagonism of Activin A receptor type IIB, the main cell surface receptor for activin A, has been reported to suppress cachexia and prolong survival in a mouse model (26). This is consistent with the role of activin A in regulating skeletal muscle degradation, suggesting that activin A released from cancer tissues may be associated with the sarcopenia observed in numerous patients with CRC. However, in the present study, no significant correlation between tumor activin A expression levels and SMI was obtained. One possible explanation is that SMI is strongly affected by age and sex, with higher SMIs observed in males and young adults compared with those observed in females and older individuals, respectively (27). In addition, Loumaye *et al* (28) reported that the blood level of activin A was associated with skeletal muscle density in patients with CRC or lung cancer and high blood levels were associated with poor prognosis. Zhong *et al* (29) reported that serum activin A secreted by pancreatic adenocarcinoma cells was associated with cachexia and poor prognosis. Thus, activin A secreted by tumor cells and other tissues may be involved in the regulation of skeletal muscle mass.

The present study suggested that high expression of activin A was associated with poor prognosis but not with SMI, which suggested that any contribution of activin A to CRC would occur via alternative mechanisms. Indeed, exposure of two CRC cell lines to exogenous activin A directly enhanced the proliferation, invasion and migration of the cells, while knockdown of endogenous activin A had the opposite effects. Furthermore, IHC staining suggested that activin A protein was detected predominantly in the cytoplasm, suggesting that it may be secreted by the CRC cells. These data indicated that CRC cells may both secrete activin A and respond to extracellular activin A, and it may be speculated that activin A therefore functions as both an autocrine and paracrine modulator of CRC malignancy.

Matsuzaki (30) reported that activin A is involved in the metastasis and invasion of cancer cells by promoting c-Myc transcription factor activity and matrix metalloproteinase-9 production via its effects on SMAD signaling. The mechanism by which activin A exposure and knockdown affects the behavior of the CRC cells assessed in the present study remains to be elucidated; however, it is reasonable to assume that it may have effects on SMAD pathway activity. The results of the present study support those of previous reports indicating that activin A contributes to the malignant behaviors of CRC cell lines, including their proliferation, migration and invasion.

The current study has certain limitations, including its retrospective design and the fact that it was performed at a single institution. However, the association between activin A expression in CRC tumors and OS was validated using a dataset from the TCGA. Furthermore, only SMI was used as an indicator of sarcopenia, although there are other indicators of sarcopenia. However, the method using abdominal CT scan was considered to be the best means of evaluating sarcopenia in patients with gastrointestinal cancer, as CT has the great advantage of being routinely performed for staging and follow-up of cancer. Furthermore, Albano *et al* (31) reported that CT is probably the easiest and most promising modality for the evaluation of sarcopenia. In addition, there are certain reports on the prognosis of CRC (9,10). Furthermore, the results of the *in vitro* experiments were also confirmed with clinical samples. The effect of activin A should be evaluated in a dose-dependent manner. In additional invasion and migration assays, treatment of LoVo and SW620 cells with 10 ng/ml activin A significantly increased their invasive ability compared with treatment with 100 ng/ml activin A; activin A did not increase invasion or migration in a dose-dependent manner (data not shown). These findings may be associated with activin A receptors and their nuclear translocation. Another limitation is that activin A secreted by the CRC cell lines into the culture medium was not removed prior to the experiments, which may have affected the results. However, given that exogenous activin A was added at a high concentration and that the opposite effects on cell phenotypes were observed upon activin A knockdown, it may be assumed that activin A secreted by the tumor cells would have only had a minor effect on the results.

In conclusion, the present study demonstrated that activin A promotes the proliferation, invasion and migration of CRC cell lines and that high expression of activin A in tumor tissues correlates with poor prognosis in patients with CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

YM and HB designed and directed the study. ND performed the laboratory experiments and collected all the clinicopathological data. YH, RT and YS assisted with the collection of clinicopathological data. HS, TI, YB, NY supported the experiments. ND and YM were responsible for the statistical analysis and wrote the manuscript. TI, YB, NY and HB supervised the study. ND, YM and HB confirmed the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

This retrospective and observational study was approved by the institutional ethics committee of Kumamoto University Hospital (14 June 2019/approval no. 1047) and performed in accordance with the Declaration of Helsinki from 1975. Informed consent for sample use was obtained from the patients and families according to institutional review board protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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