cell cycle repair. The chemosensitivity and radiosensitivity of several types of cancers have been demonstrated to be *TP53*-dependent in the number of preclinical studies [16, 18-20]. Further, the presence of *TP53* mutations significantly decreased the radiation-induced senescence in head and neck squamous cell lines [19]. In addition, *TP53* mutations and overexpression were associated with the response to neoadjuvant treatment in breast cancer patients in the clinical setting [21]. Recently, the *TP53* genotype was reported to be associated with the survival after adjuvant treatment in female colon cancer patients in a clinical study (CALGB89803) [22].

We found that there was an increased population of tumors with *TP53* mutations and *TP53* overexpression in the post-treatment samples. When we compared the mutation profiles between the pre- and post-treatment samples, the same types of tumor suppressor gene mutations were detected (Figure 1). Based on that finding, we speculated that chemoradiation provides a physiological selective pressure for the expansion of *TP53* mutant tumor variants in residual cancerous tissue. An increased frequency of *TP53* mutants and an increased intensity of p53 immunostaining were consistently observed in post-treatment samples compared with the respective pre-treatment samples in five of the nine cases. No change in the *TP53* mutation frequency and p53 immunostaining were observed between the pre- and post-treatment samples in one case (No. 2). In the other two cases, a higher frequency of *TP53* mutations was observed in the pre-treatment samples compared to the post-treatment samples, whereas no differences were observed by p53 immunostaining.

On the other hand, it is not surprising that there was a discrepancy in the changes in the *TP53* status between pre- and post-treatment samples at the genome, transcript and protein expression levels, because *TP53* expression is regulated, at least partly, by post-translational regulation. In a series of 67 breast tumors, 19% had *TP53* gene mutations, 40% had a positive *TP53* IHC result and 12% had both [21].

In our study, we found an increased population of tumor cells with *FBXW7* mutations after chemoradiation in two of the three cases examined. *FBXW7* (also known as Fbw7, Sel-10, hCdc4 or hAgo), an F-box protein subunit of an SCF-type ubiquitin ligase complex, induces the degradation of positive regulators of the cell cycle, such as c-Myc, c-Jun, cyclin E and Notch. *FBXW7* is often mutated in a subset of human cancers including CRC [23, 24]. Thus, *FBXW7* is also a critical tumor suppressor gene. Because *FBXW7* also participates in the cell cycle exit to, and the reentry from, the G_0 phase, it is a candidate molecular therapeutic target in intractable carcinoma cases that are resistant to combined modality therapies [25-27]. Onoyama *et al.* reported consecutive roles for *TP53* and *FBXW7* in the carcinogenesis of solid tumors *in vivo*. Moreover, a comparison of four groups classified according to the *FBXW7* and *TP53* status revealed a worse prognosis for double inactivation mice compared to the other subgroups [27]. The clinical significance of *FBXW7* in human solid cancers has been diversely reported, and lower expression of *FBXW7* was associated with chemoresistance in one study [28].

In our study, we also found an increased population of tumor cells with *APC* mutations after chemoradiation in all three cases examined. The adenomatous polyposis coli (APC) tumor suppressor is the most commonly mutated gene in colorectal cancers. In addition to its effects on the Wnt/β-catenin signaling pathway, APC also regulates other processes in a Wnt/β-catenin-independent manner, such as the apical-basal polarity, microtubule networks, cell cycle progression, DNA replication and repair, apoptosis and cell migration [29].

A limitation of the present study is the small sample size which may reduce the power of the study. Still, we were able to detect differences in the expression of some genes in samples before and after treatment, albeit, the data must be interpreted cautiously since it is difficult to arrive to a solid conclusion because of the limited sample size. Nevertheless, we have shown that in principle, gene expression analysis targeting RNA sequencing of FFPE samples is feasible using NGS.

In summary, the results from the present study suggest the possibility that chemoradiotherapy induces plasticity and therapeutic escape in cancer cells and is manifested by the differential expression of genes from resistant cancer cells present in residual colorectal tumors. The present sample set has allowed us to explore possible relationship of *TP53* between responders and non-responders for chemoradiotherapy and revealed differences in the mutation rates of various genes, including *TP53*. In order to strengthen our hypothesis, we included the data of gene expression, protein expression (IHC) from the same sample set. To conclude, this study provides the foundation to warrant future large scale studies to validate *TP53* alterations after chemoradiation in colorectal cancer patients.

MATERIALS AND METHODS

Patients and samples

In order to elucidate biological differences in response to chemoradiotherapy, we compared differences between an equivalent number of samples of responders and non-responder cases. A total of 20 rectal cancer patients who chose to receive preoperative chemoradiotherapy at the Department of Surgical Oncology, the University of Tokyo Hospital, between 2006 and 2011 were analyzed retrospectively. Biopsy samples were taken from the rectal cancers during colonoscopic

examinations before preoperative chemoradiotherapy was performed, and these were classified as "pre-treatment samples". All patients underwent surgical resection of the rectal cancer, and the surgically resected specimens were classified as "post-treatment samples". Both the pre-treatment and post-treatment samples were fixed in 10% formalin and embedded in paraffin. Informed consent was obtained from all patients for the collection of specimens for future analyses, and the study protocol was approved by the Ethics Committee of Tokyo University Faculty of Medicine and the Kinki University Faculty of Medicine. The characteristics of the patients included in this study are summarized in Supplemental Table 2.

All patients received a total dose of 50.4 Gy of radiation, given in 28 fractions over six weeks. Tegafur-uracil (300–500 mg/day) and leucovorin (75 mg/day) were given concomitantly with radiotherapy. Standardized curative resection was performed six weeks after the completion of chemoradiotherapy. The response to chemoradiotherapy was determined by a histopathological examination of the surgically resected specimens based on a semiquantitative classification system defined by the Japanese Society for Cancer of the Colon and Rectum. In brief, grade 0 indicated no tumor cell necrosis or degeneration; grade 1 indicated tumor cell necrosis or degeneration in less than two-thirds of the entire lesion; grade 2 indicated prominent tumor cell necrosis or degeneration in more than two-thirds of the entire lesion, but with viable tumor cells remaining and grade 3 indicated that there were no viable tumor cells (Japanese Classification of Colorectal Carcinoma. Kanehara & Co., Ltd, Tokyo, Japan). Tumors were classified as "responders" when assigned regression grade 2 or 3, and "non-responders" when assigned grade 0 or 1. As noted above, the FFPE pre-treatment samples were obtained by biopsy. The post-treatment samples were obtained by surgical resection after chemo-radiation in non-responder cases.

DNA and RNA extraction

Collected FFPE specimens were subjected to a histological review, and only those containing sufficient tumor cells (at least 75% tumor cells) as determined by hematoxylin and eosin (H&E) staining were subjected to DNA/RNA extraction. DNA and RNA were purified using an Allprep DNA/RNA FFPE kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quality and quantity of the DNA/RNA were verified using the NanoDrop 2000 device (Thermo Scientific Wilmington, DE), PicoGreen dsDNA assay kit (Life Technologies) and the RiboGreen RNA assay kit (Life Technologies). The extracted DNA/RNA was stored at −80°C until the analysis.

RNA sequencing

For RNA sequencing, PCR primers were designed using the Ion AmpliSeq Designer software program (Life Technologies). The Ion AmpliSeq RNA Library Kit (Life Technologies) was used to construct the RNA library according to the manufacturer's instructions. Briefly, 20 ng of total RNA were reverse transcribed with the SuperScript III enzyme, followed by PCR amplification. The Ion Xpress Barcode adapters (Life Technologies) were ligated into the PCR products and purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Purified libraries were pooled and sequenced on an Ion Torrent PGM (Life Technologies) using the Ion PGM 200 Sequencing Kit v2 and the Ion 318 v2 Chip Kit.

DNA sequencing

We used 20 ng of DNA for the multiplex PCR amplification using the Ion AmpliSeq Library Kit and the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) according to the manufacturer's instructions. The Ion Xpress Barcode Adapters (Life Technologies) were ligated into the PCR products and purified with Agencourt AMPure XP beads (Beckman Coulter). Purified libraries were pooled and sequenced on an Ion Torrent PGM device (Life Technologies) using the Ion PGM 200 Sequencing Kit v2 and the Ion 318 v2 Chip Kit.

DNA sequencing data were accessed through the Torrent Suite v.3.4.2 software program. Reads were aligned against the hg19 human reference genome, and variants were called using the variant caller v 3.6. Raw variant calls were filtered out using the following annotations: homozygous and heterozygous variants, quality score of <100, depth of coverage <19.

Immunohistochemical analysis

Immunohistochemical analyses were performed on nine paired samples from non-responder patients. Deparaffinized and rehydrated sections were heated in a microwave oven for seven three-minute cycles in citrate buffer to retrieve antigens, and were cooled for 15 min at room temperature. The endogenous peroxidase activity was inhibited by incubation of the samples with 0.3% hydrogen peroxidase in methanol for 20 min at room temperature. After blocking the non-specific reactions with 10% normal rabbit serum, the sections were first incubated with an anti-p53 antibody (mouse monoclonal antibody DO 7; Novocastra) overnight at a dilution of 1:100. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 min, and next with streptavidin–peroxidase complex

(Histofine SAB-PO Kit, Biogenex Laboratories) for 15 min. The sections were carefully rinsed with several changes of phosphate-buffered saline (PBS) between each step of the procedure. The color was developed with diaminobenzidine. The sections were lightly counterstained with hematoxylin and mounted. Negative controls were obtained by replacing the primary antibody with PBS. The specimens immunostained for p53 were independently evaluated by two authors (S.K and T.W) who received training of pathological diagnosis without knowledge of the clinicopathological features. Nuclear staining of cancer cells was evaluated according to both the stained area and the intensity of the nuclear-stained cancer cells observed under 25× magnification. We scored the intensity of the nuclear-stained cancer cells into 3 categories: none; 0, moderate; 1+, or strong; 2+, respectively.

Statistical analysis

A non-parametric statistical method (Mann-Whitney U-test) was used for comparisons between the responders and non-responders. The paired t-test was used for comparisons between pre-treatment and post-treatment samples in the non-responders. Statistical analyses were carried out using the JMP software program (version 10; SAS Institute, Cary, NC). A value of $P < 0.05$ was considered to be statistically significant.

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Conflict of interest

No potential conflicts of interest were disclosed by all of the authors.

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Impact of short-term exposure to fine particulate matter on emergency ambulance dispatches in Japan

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ABSTRACT

Background Evidence of an association between fine particulate matter ($PM_{2.5}$) and morbidity is limited in Asia. We used a case-crossover design to evaluate the association between short-term exposure to $PM_{2.5}$ and emergency ambulance dispatches (as a proxy of acute health outcomes), and to calculate the extent to which a 10 μg/m^3 decrease in $PM_{2.5}$ concentrations would reduce the number of ambulance dispatches.

Methods We used data on emergency ambulance dispatches in Fukuoka City, Japan between 2005 and 2010. Emergency ambulance services are publicly funded and cover the entire city. After excluding ambulance dispatches related to external injuries and pregnancy/childbirth, we analysed data on the remaining 176 123 dispatches. We also collected records of daily concentrations of $PM_{2.5}$ from one ambient air pollution monitoring station. ORs per 10 μg/m^3 increase in $PM_{2.5}$ were estimated using conditional logistic regression controlled for ambient temperature and relative humidity.

Results During the study period, the average daily concentration of $PM_{2.5}$ was 20.3 μg/m^3. Exposure to $PM_{2.5}$ was associated with emergency ambulance dispatches in general (lag0–1; OR=1.008 (95% CI 1.002 to 1.014)) and with dispatches due to respiratory diseases (lag0–1; OR=1.027 (1.007 to 1.048)). No association was observed for dispatches due to cardiovascular diseases. We estimated that a 10 μg/m^3 decrease in $PM_{2.5}$ concentrations would have led to approximately 260 (estimated range=70–460) fewer ambulance dispatches in Fukuoka for 2012.

Conclusions Providing further evidence on the short-term health effects of $PM_{2.5}$ exposure, we found that exposure was associated with an increased number of emergency ambulance dispatches. The effect was, however, relatively small.

INTRODUCTION

There is growing evidence that exposure to particulate matter harms human health,[1] even though the effects may seem relatively small in comparison with those of major risk factors like smoking. However, as more and more populations are exposed to particulate matter, it is becoming a global public health burden. A systematic analysis for the Global Burden of Disease Study 2010 put fine particulate matter ($PM_{2.5}$, ie, fine particulate matter collected with a sampler with a 50% cut-off point of 2.5 μm) in the top 10 causes of disease burden worldwide, accounting for 3.2 million deaths in 2010.[2]

There is limited evidence on the health effects of $PM_{2.5}$ exposure in Japan, because air quality standards were based on suspended particulate matter measurements (particles with diameters of less than 10 μm) until $PM_{2.5}$ was added as a criterion in 2009. In other Asia countries, evidence of an association between exposure to $PM_{2.5}$ and morbidity is also more limited than it is in Europe and the USA. It is also clear from the literature that epidemiological evidence collected in Western countries cannot necessarily be extrapolated to Asian populations. In addition, the health effects of $PM_{2.5}$ have been reported to differ according to its chemical composition, which is affected by geographic location.[3] Therefore, the health effects of $PM_{2.5}$ exposure needs further study in Asia. In Japan, this association has become a matter of national concern because of high concentrations found in air pollution drifting periodically into the country from other East Asian nations recently.[4 5] Information on the short-term health effects of $PM_{2.5}$ that are closely connected to everyday life is urgently needed.

The purpose of the present time-stratified case-crossover study was to examine whether short-term increases in $PM_{2.5}$ concentrations are associated with the number of emergency ambulance dispatches in Japan. There is precedent for using ambulance dispatch data to examine the health effects of $PM_{2.5}$[6 7]; such data are helpful in assessing acute health events related to respiratory and cardiovascular diseases, which are the main focus of epidemiological studies on $PM_{2.5}$. Quantification of the public health impact of $PM_{2.5}$ exposure is important, so we also calculated the extent to which a 10 μg/m^3 decrease in $PM_{2.5}$ concentrations would reduce the number of ambulance dispatches.

MATERIALS AND METHODS

Study area

This study was carried out in Fukuoka City, Fukuoka Prefecture, one of the biggest cities in Japan (see online supplementary figure), with a population of approximately 1.5 million, and a moderate climate (average annual temperature c.17°C). Fukuoka is in Kyushu (130°24′E, 33°35′N), the closest of Japan's main islands to the Asian continent, making it susceptible to transboundary air pollution.[4 8] Owing partly to this susceptibility, $PM_{2.5}$ concentrations in Fukuoka are relatively high compared with other Japanese cities; the range of daily mean concentrations is also wide.[9]

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Environmental data

We obtained data on $PM_{2.5}$ from Fukuoka City. The tapered element oscillating microbalance (TEOM) method was used to measure hourly concentrations of $PM_{2.5}$ almost every day during the 2191-day study period (measurements were unavailable for 1.3% of the total days) at one ambient air pollution monitoring station selected as representative of the urban background in Fukuoka, and daily mean concentrations were calculated. TEOM measurements correlate well with those obtained by the federal reference method.[10]

Data on other pollutants (nitrogen dioxide (NO_2), photochemical oxidants (Ox) and sulfur dioxide (SO_2)) were obtained from the National Institute for Environmental Studies' atmospheric environment database. Ox is defined as mixtures of ozone and other secondary oxidants generated by photochemical reactions, and is considered to be a proxy for ozone. Hourly data from eight monitoring stations in Fukuoka were collected, and daily mean concentrations of NO_2 and SO_2 were calculated across the monitoring stations, along with average daily maximum 8 h mean concentrations of Ox.

Meteorological data were obtained from the Japan Meteorological Agency. Hourly measurements were used to calculate daily mean ambient temperatures and relative humidity. Data on weekly influenza incidence were obtained from the Japan National Institute of Infectious Diseases. Influenza epidemic week was defined as the week when the weekly number of influenza cases was above the 90th centile of distribution during the study period or not,[11] so influenza was a dichotomised variable as epidemic week or not.

Information on emergency ambulance dispatches

In Japan, emergency medical services are provided free of charge by local governmental fire defence headquarters,[12 13] and citizens can call ambulances via the emergency number 119.

We obtained data on 307 711 emergency ambulance dispatches covering the entire city of Fukuoka between 1 January 2005 and 31 December 2010. After excluding dispatches related to external injuries and pregnancy/childbirth, we were left with data on 176 123 dispatches (57.2%) for analysis. Records of the initial diagnoses made at the emergency departments where the transported patients were treated were used to code the causes of the ambulance dispatches according to the International Classification of Diseases, 10th revision (ICD-10); one medical doctor (TM) did the coding. Since there is evidence of an association between $PM_{2.5}$ and cardiopulmonary diseases,[1] we defined ambulance dispatches related to respiratory diseases (ICD-10: J00-99) and cardiovascular diseases (ICD-10: I00-99), which accounted for 9% (n=15 857) and 18.1% (n=31 837) of the total, respectively, as cause-specific. The ratio of ambulance dispatches due to respiratory and cardiovascular diseases was similar to that found in an earlier study in another Japanese city.[14]

We submitted the study protocol to the Ethical Review Board of the National Institute for Environmental Studies for approval but were told by the Board that approval was not required because the study data did not include individual identifiers.

Statistical analyses

The case-crossover design used in this study was developed to assess the acute effects of transient exposure. The design has the ability to control for time-invariant characteristics such as age and sex, because within-subject comparisons are made between case periods and control periods. Case periods in this study were defined as the days of emergency ambulance dispatches, and the control periods (3–4 per case) were selected on corresponding days of the week within the same calendar month. For example, if an ambulance was dispatched on 15 October, four control days were assigned: 1, 8, 22 and 29 October. This control selection strategy is called 'time-stratification', and is designed to adjust for time-dependent confounding within strata-time by methods.[15]

Conditional logistic regression models were used to estimate the adjusted ORs and 95% CIs of emergency ambulance dispatches associated with 10 $\mu g/m^3$ increases in $PM_{2.5}$ concentrations, after including the mean ambient temperature (a natural spline with 4° of freedom) and relative humidity from the case day (lag0) to 3 days prior (lag3). To evaluate the lag effect of $PM_{2.5}$, we applied a lag-stratified distributed lag model,[16] entering the average concentrations of $PM_{2.5}$ during the case day (lag0) to 1 day preceding it (lag1) (lag0–1), during lag2–lag3 (lag2–3) and during lag4–lag6 (lag4–6) simultaneously. To confirm the robustness of $PM_{2.5}$ effect estimates against potential confounding by NO_2, Ox and SO_2, we constructed two-pollutant models adjusted for the mean concentrations of each copollutant (lag0–3). Pearson's correlations coefficients between $PM_{2.5}$ and the other pollutants were 0.43 for NO_2, 0.41 for Ox and 0.60 for SO_2 (see online supplementary table S1). Although these correlations by season were different, particularly regarding Ox, there was no statistical evidence in two-pollutant models that the association between $PM_{2.5}$ and ambulance dispatches varied according to season. We also performed stratified analyses by age (0–19 years, 20–64 years and 65 years or older) and sex to examine the effects by subgroup, and a statistical check for interaction by age strata and sex using the likelihood ratio test. Similar analyses were repeated to investigate other pollutants. Furthermore, several sensitivity analyses were performed. First, because of possible bias in control selection, we excluded participants transported to hospitals on national holidays. Second, we investigated whether any association between $PM_{2.5}$ and ambulance dispatches was detectable when $PM_{2.5}$ concentrations were lower than those of air quality standards stipulated by the Japanese Ministry of the Environment[17] and US Environmental Protection Agency[18] (daily mean, 35 $\mu g/m^3$), and by the WHO[19] (daily mean, 25 $\mu g/m^3$): analyses were restricted to participants exposed to $PM_{2.5}$ concentrations below those values. Third, we additionally adjusted for influenza epidemics. And fourth, to check for possible confounding by the effects of prolonged low temperatures, a significant factor in respiratory disease,[20] we adjusted for temperature at lag0–14.[11]

To illustrate the public health impact, we estimated the reduction in the number of annual emergency ambulance dispatches attributable to a 10 $\mu g/m^3$ decrease in the daily $PM_{2.5}$ concentrations. In accordance with a modified method for calculating the population attributable fraction,[21] the annual reduction was defined as $N \times (OR-1)/OR$, where N was the number of annual ambulance dispatches excluding those related to external injuries and pregnancy/childbirth. By multiplying the total number of ambulance dispatches by 0.572 (57.2%), we thus assumed approximately 33 000 ambulance dispatches in Fukuoka for 2012[22] as N. We also estimated the corresponding reduction in administrative expenditure based on a rate of 7600 Japanese yen per ambulance dispatch[23] and using exchange rates of 100 yen=$1=€0.7.

All statistical analyses were performed with Stata V.11 (Stata Corporation, College Station, Texas, USA), and statistical tests were two-tailed with significance levels set at $p<0.05$.

RESULTS

Of the 176 123 emergency ambulance dispatches investigated, 9.3% were for persons aged 0–19 years, 45.3% for people aged

Table 1 Summary of meteorological factors and daily mean concentrations of air pollutants from January 2005 to December 2010 in Fukuoka, Japan

Pollutants	Number of days	Mean (SD)	Min	IQR	Max	Number of days over air quality standards of $PM_{2.5}$ (%)	
						Japan (35 μg/m³)	WHO (25 μg/m³)
$PM_{2.5}$ (μg/m³)	2162	20.3 (11.2)	2.9	13.3	83.0	211 (9.8)	670 (31.0)
NO_2 (ppb)	2191	14.0 (6.4)	1.9	8.4	40.2		
Ox (ppb)*	2191	44.1 (15.5)	2.7	19.8	77.0		
SO_2 (ppb)	2191	3.5 (1.6)	0.7	2.0	12.8		
Temperature (°C)	2191	17.4 (7.9)	0.7	13.8	31.8		
Relative humidity (%)	2191	65.0 (11.1)	26.6	15.5	94.3		

*Daily maximum 8 h mean concentrations.
NO_2, nitrogen dioxide; Ox, photochemical oxidants; $PM_{2.5}$, fine particulate matter; SO_2, sulfur dioxide.

20–64 years and 45.4% for people aged 65 years or older; 86 127 dispatches (48.9%) were for men. A summary of meteorological factors and daily mean concentrations of air pollutants is shown in table 1. The average $PM_{2.5}$ concentration over the study period was 20.3 μg/m³, and concentrations exceeded Japanese air quality standards on 211 days (9.8%).

Figure 1 shows the association between $PM_{2.5}$ exposure and emergency ambulance dispatches. $PM_{2.5}$ exposure was associated with increased ambulance dispatches for lag0–1 (OR=1.008 per 10 μg/m³, 95% CI 1.002 to 1.014). When the subjects transported to hospitals on national holidays were excluded, similar results were obtained (OR=1.008, 95% CI 1.002 to 1.014). We restricted analyses to subjects exposed to $PM_{2.5}$ concentrations below those stipulated by air quality standards. Although the point estimations changed slightly, those 95% CIs showed considerable overlap (OR=1.010 (95% CI 1.001 to 1.019) for ≤35 μg/m³, OR=1.007 (0.994 to 1.020) for ≤25 μg/m³). Including influenza epidemics in the model did not affect our results (OR=1.008, 95% CI 1.002 to 1.014; see online supplementary table S2). In the two-pollutant models, the OR estimates for lag0–1 were similar (figure 1). The OR for lag0–1 was used to calculate the reduction in annual ambulance dispatches attributable to a 10 μg/m³ decrease in daily $PM_{2.5}$ concentrations. The estimated reduction was approximately 260 (estimated range=70–460) dispatches in Fukuoka for 2012, with a correspondingly reduced administrative expenditure of approximately 2 million yen (c. $20 000/€14 000).

Table 2 shows the results of cause-specific emergency ambulance dispatches according to age strata and sex. $PM_{2.5}$ (lag0–1) was associated with an elevated risk of ambulance dispatches due to all causes in those aged 65 years or older (OR=1.012 per 10 μg/m³, 95% CI 1.003 to 1.021) and in men (OR=1.011, 95% CI 1.002 to 1.020); however, no statistical evidence for interaction by age strata or sex was observed. In the cause-specific analyses, the OR for respiratory diseases was 1.027 at lag0–1 (95% CI 1.007 to 1.048) in the overall analysis. We confirmed the robustness of this finding by adjusting for temperature at lag0–14 (see online supplementary table S3). In contrast, we did not find any associations for cardiovascular diseases.

Table 3 presents the associations between other air pollutant concentrations and emergency ambulance dispatches. Exposure to copollutants was not associated with ambulance dispatches due to all causes. However, positive associations were observed between NO_2 and SO_2 concentrations and respiratory diseases

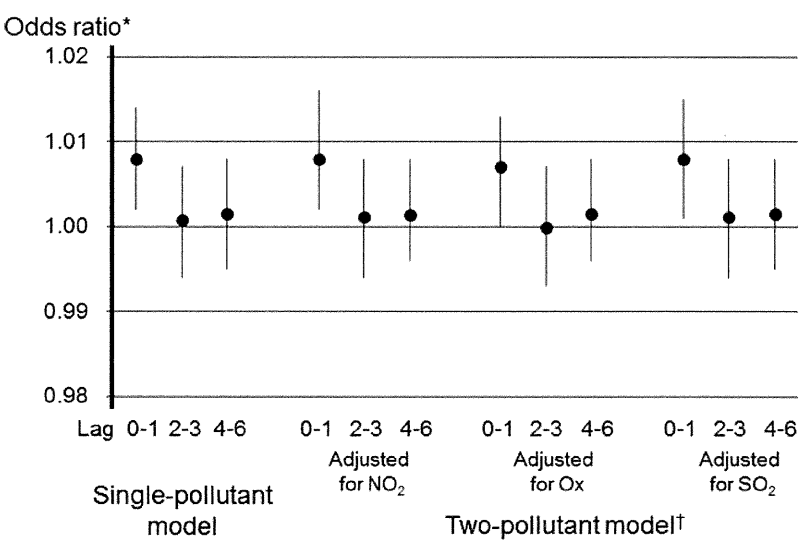

Figure 1 ORs (black circles) of emergency ambulance dispatches per 10 μg/m³ increase in fine particulate matter ($PM_{2.5}$). Error bars indicate 95% CIs.

NO_2, nitrogen dioxide; Ox, photochemical oxidants; SO_2, sulfur dioxide.
*We entered the average concentrations of $PM_{2.5}$ during lag0-lag1 (lag0-1), during lag2-lag3 (lag2-3) and during lag4-lag6 (lag4-6) simultaneously in the model, and also controlled for 4-day average ambient temperature and relative humidity.
†Additionally adjusted for 4-day average concentrations of each air pollutant.

Table 2 ORs and 95% CIs of cause-specific emergency ambulance dispatches per 10 μg/m^3 increase in fine particulate matter ($PM_{2.5}$) by age strata and sex

	All causes				Respiratory diseases				Cardiovascular diseases			
	n	OR*	95% CI	p Value†	n	OR*	95% CI	p Value†	n	OR*	95% CI	p Value†
Total												
Lag0–1	176 123	1.008	1.002 to 1.014		15 857	1.027	1.007 to 1.048		31 837	1.002	0.987 to 1.016	
Lag2–3		1.001	0.994 to 1.007			1.003	0.982 to 1.025			0.998	0.983 to 1.013	
Lag4–6		1.002	0.995 to 1.008			1.009	0.987 to 1.031			0.991	0.976 to 1.007	
Age (years)												
<20												
Lag0–1	16 415	1.011	0.991 to 1.031	0.59	1816	0.990	0.932 to 1.052	0.18	196	0.977	0.816 to 1.170	0.47
Lag2–3		0.999	0.978 to 1.020			1.050	0.988 to 1.116			0.909	0.740 to 1.117	
Lag4–6		0.991	0.969 to 1.013			0.977	0.913 to 1.046			0.914	0.750 to 1.115	
20–64												
Lag0–1	79 734	1.004	0.995 to 1.013		4325	0.993	0.955 to 1.032		10 001	0.998	0.973 to 1.023	
Lag2–3		1.002	0.993 to 1.012			1.044	1.002 to 1.088			0.994	0.967 to 1.021	
Lag4–6		1.006	0.997 to 1.016			1.015	0.973 to 1.060			0.990	0.962 to 1.018	
≥65												
Lag0–1	79 974	1.012	1.003 to 1.021		9716	1.048	1.022 to 1.076		21 640	1.004	0.986 to 1.021	
Lag2–3		0.999	0.990 to 1.009			0.977	0.950 to 1.005			1.001	0.982 to 1.019	
Lag4–6		0.999	0.989 to 1.009			1.010	0.982 to 1.040			0.992	0.974 to 1.012	
Sex												
Men												
Lag0–1	86 127	1.011	1.002 to 1.020	0.63	8405	1.030	1.002 to 1.059	0.93	17 116	1.006	0.987 to 1.026	0.26
Lag2–3		1.001	0.992 to 1.010			1.006	0.977 to 1.037			1.011	0.991 to 1.033	
Lag4–6		0.996	0.987 to 1.006			1.009	0.979 to 1.041			0.992	0.971 to 1.013	
Women												
Lag0–1	89 996	1.005	0.997 to 1.014		7452	1.024	0.994 to 1.054		14 721	0.996	0.975 to 1.018	
Lag2–3		1.001	0.992 to 1.010			1.000	0.969 to 1.032			0.982	0.961 to 1.005	
Lag4–6		1.007	0.997 to 1.016			1.008	0.976 to 1.042			0.990	0.968 to 1.013	

*We entered the average concentrations of $PM_{2.5}$ during lag0-lag1 (lag0–1), during lag2-lag3 (lag2–3) and during lag4-lag6 (lag4–6) simultaneously in the model, and also controlled for 4-day average ambient temperature and relative humidity.
†Statistical interaction between $PM_{2.5}$ and age strata and sex was tested by using a likelihood ratio test.

for lag2–3, with estimated ORs of 1.047 per 10ppb (95% CI 1.005 to 1.091) and 1.196 per 10 ppb (95% CI 1.001 to 1.429), respectively. With regard to NO_2, the statistically significant association was found in those aged 19 years or younger (OR=1.162, 95% CI 1.028 to 1.313).

DISCUSSION

Observed association between short-term exposure to $PM_{2.5}$ and emergency ambulance dispatches was robust to adjustment for copollutants and was independent of influenza epidemics. An estimated increase in ambulance dispatches of 0.8% per

Table 3 Associations of other air pollutants with emergency ambulance dispatches

	All causes (n=176 123)		Respiratory diseases (n=15 857)		Cardiovascular diseases (n=31 837)	
	OR*	95% CI	OR*	95% CI	OR*	95% CI
NO_2 (per 10 ppb)						
Lag0–1	1.005	0.993 to 1.017	1.032	0.993 to 1.072	0.989	0.963 to 1.016
Lag2–3	1.001	0.988 to 1.014	1.047	1.005 to 1.091	0.996	0.967 to 1.026
Lag4–6	1.005	0.992 to 1.019	0.996	0.953 to 1.040	0.991	0.961 to 1.022
Ox (per 10 ppb)						
Lag0–1	1.005	0.999 to 1.011	1.011	0.991 to 1.030	1.004	0.990 to 1.018
Lag2–3	1.004	0.998 to 1.010	1.005	0.984 to 1.026	0.997	0.983 to 1.012
Lag4–6	0.998	0.992 to 1.004	1.009	0.988 to 1.030	0.994	0.979 to 1.008
SO_2 (per 10 ppb)						
Lag0–1	1.026	0.973 to 1.081	1.095	0.924 to 1.297	1.001	0.886 to 1.132
Lag2–3	1.016	0.961 to 1.074	1.196	1.001 to 1.429	0.979	0.862 to 1.113
Lag4–6	1.035	0.976 to 1.097	1.035	0.855 to 1.253	1.028	0.898 to 1.177

*We entered the average concentrations of each pollutant during lag0-lag1 (lag0–1), during lag2-lag3 (lag2–3) and during lag4-lag6 (lag4–6) simultaneously in the model, and also controlled for 4-day average ambient temperature and relative humidity.
NO_2, nitrogen dioxide; Ox, photochemical oxidants; SO_2, sulfur dioxide.

10 μg/m^3 rise in $PM_{2.5}$ appeared to be similar to that reported in an earlier case-crossover analysis of all-cause mortality in Fukuoka City,[9] though different outcomes made it difficult to directly compare our results with the earlier findings. We found no statistical evidence of interaction by age strata. A recent meta-analysis presented the higher particulate matter (aerodynamic diameter ≤10 μm (PM_{10}))-associated mortality risk in people aged 65 years or older,[24] so further research into age-specific differences is needed. In this meta-analysis, a 10 μg/m^3 increase in PM_{10} exposure was associated with 0.28% (95% CI 0.11 to 0.44) and 0.34% (95% CI 0.19 to 0.49) increases in risk of death for men and women, respectively.[24] The overlap of 95% CIs means a less pronounced difference between men and women, suggesting consistency between our findings and the results of this meta-analysis.

As far as we know, this is the first study of the impact of exposure to $PM_{2.5}$ on emergency ambulance dispatches. By our estimate, a 10 μg/m^3 decrease in $PM_{2.5}$ concentrations would have reduced the number of ambulance dispatches in Fukuoka by approximately 260 (estimated range=70–460) in 2012. As ambulance dispatches are not to be related to exposure to $PM_{2.5}$ might remain in the all-cause dispatches, we should be cautious about possible overestimation. As our results show, the increased health risk of $PM_{2.5}$ exposure is relatively small. However, since populations are aging rapidly all over the world, increasing vulnerability to $PM_{2.5}$ exposure is expected. Japan has the longest life expectancy at birth worldwide, and the number of ambulance dispatches is estimated to exceed 6 million annually by 2023 or 2024.[13] From the viewpoint of public health, the sheer number of people subject to $PM_{2.5}$ exposure means that even a small increased health risk is likely to have a financial impact on the emergency ambulance system. The estimated association between $PM_{2.5}$ exposure and ambulance dispatches tended to be similar when analyses were restricted to subjects exposed to $PM_{2.5}$ concentrations below those stipulated in Japanese air quality standards, so efforts should be made to reduce $PM_{2.5}$ exposure as far as possible.

With respect to cause-specific emergency ambulance dispatches, we observed an association between short-term $PM_{2.5}$ exposure and increased risk for respiratory diseases. This finding is biologically plausible, because the respiratory system is the first system affected by contact with $PM_{2.5}$; $PM_{2.5}$ causes lung inflammation.[1] Also, this does not contradict the results of past epidemiological studies. A study in Linz, Austria showed that exposure to $PM_{2.5}$ tended to increase the risk of emergency ambulance dispatches for respiratory diseases at lag0 (per cent increase with a 10 μg/m^3 increase=2.1%, 95% CI −2.1 to 6.3).[6] In studies that used emergency department visits as a morbidity indicator, the point estimates for respiratory diseases were above unity, with or without statistical significance.[25–30]

In contrast with respiratory diseases, we did not observe any association between $PM_{2.5}$ exposure and cardiovascular-related emergency ambulance dispatches, although $PM_{2.5}$ exposure is strongly suspected to be a risk factor for cardiovascular diseases[1]: an increase risk of cardiovascular emergencies was shown in relation to $PM_{2.5}$ exposure in Linz, Austria.[6] However, not all studies report statistically significant associations between $PM_{2.5}$ and cardiovascular disease-related mortality or morbidity probably because of demographic and geographic differences.[31 32] The null association in our present study might be explained by difference in the pattern of cardiovascular diseases between Japan and Western countries: in Japan, the incidence of stroke is much higher than that of coronary heart disease,[33] and while the association between $PM_{2.5}$ and coronary heart disease has been documented, that between $PM_{2.5}$ and stroke is still unclear.[1] Further studies in terms of cardiovascular morbidity are required in Japan.

NO_2 and SO_2 exposure was positively associated in our study with emergency ambulance dispatches due to respiratory diseases. Observed associations were only for lag2–3, which might be explained by chance. However, exposure to NO_2 and SO_2 is known to be associated with respiratory symptoms,[34] and NO_2 exposure has the potential to adversely affect lung defence mechanisms against infection.[35] In a recent panel study, short-term exposure to NO_2 was linked with airway inflammation and/or oxidative stress in healthy and asthmatic adolescents aged 14–19 years.[36] Exposure to SO_2 is known to decrease lung function and increase airway resistance and respiratory symptoms.[34] Since ambient SO_2 contributes to acid aerosol formation, the health effects of acid aerosols are also pertinent.[34] Although the biological mechanisms by which NO_2 and SO_2 exert adverse effects on the respiratory system are not fully understood, our findings are unsurprising.

One strength of our study is that the data included all emergency ambulance dispatches in Fukuoka City, so any selection bias caused by not covering all of the target subjects was negligible. In addition, we analysed approximately 176 000 cases, giving the study enough statistical power to detect even small effects of $PM_{2.5}$ exposure and to estimate the precise effects of $PM_{2.5}$ exposure on ambulance dispatch numbers. As a result, we were able to quantify the effect of $PM_{2.5}$ exposure on ambulance dispatches regarding public health impact. The study also has some limitations, however. First, all data on $PM_{2.5}$ concentrations came from a single monitoring station. While $PM_{2.5}$ concentrations are known to be spatially homogeneous,[37] exposure misclassification may have led to underestimation of the effects of $PM_{2.5}$ on ambulance dispatches. Second, the cause-specific ambulance dispatches were classified on the basis of the initial diagnoses made by emergency physicians; these diagnoses might have changed later in some cases. However, a earlier study indicates no bias in the assignment of cardiopulmonary diagnoses related to daily air pollution concentrations in emergency departments.[38] We think outcome misclassifications are likely to be non-differential, so we have no reason to suspect that using initial diagnoses affected the observed associations. Third, a possibility of residual confounding by influenza

What is already known on this subject

There is growing evidence, particularly in Europe and the USA, of an association between short-term exposure to fine particulate matter ($PM_{2.5}$) and mortality and morbidity.

What this study adds

- We showed that exposure to $PM_{2.5}$ was associated with an increased number of emergency ambulance dispatches (as a proxy of acute health outcomes) in Fukuoka City, western Japan.
- To illustrate the public health impact, we estimated the extent to which a 10 μg/m^3 decrease in $PM_{2.5}$ concentrations would reduce the number of emergency ambulance dispatches.

was left, because we did not obtain daily data on influenza incidence. A final limitation is that this study was performed in just one Japanese city, so further studies of other populations and locations are necessary to confirm the generalisability of our results.

In conclusion, by showing that exposure to $PM_{2.5}$ was associated with emergency ambulance dispatches related to respiratory diseases, but not associated with cardiovascular-related dispatches, we have provided further evidence on the short-term health effects of $PM_{2.5}$ exposure.

Contributors TM and KU specified the research question, and contributed to data collection, analysis, interpretation of results and drafting of the article. AT participated in analysis, interpretation of results and made critical revisions of the manuscript. MK and HH assisted with research design and contributed to data collection and critical revisions of the manuscript. TI assisted with research design, participated in interpretation of results and critical revisions of the manuscript. HN supervised the study and contributed to interpretation of results and critical revisions of the manuscript. All the authors approved the final version for submission.

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Original Articles

Activin signal promotes cancer progression and is involved in cachexia in a subset of pancreatic cancer

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ABSTRACT

We previously reported that activin produces a signal with a tumor suppressive role in pancreatic cancer (PC). Here, the association between plasma activin A and survival in patients with advanced PC was investigated. Contrary to our expectations, however, patients with high plasma activin A levels had a significantly shorter survival period than those with low levels (median survival, 314 days vs. 482 days, $P = 0.034$). The cellular growth of the MIA PaCa-2 cell line was greatly enhanced by activin A via non-SMAD pathways. The cellular growth and colony formation of an *INHBA* (beta subunit of inhibin)-overexpressed cell line were also enhanced. In a xenograft study, *INHBA*-overexpressed cells tended to result in a larger tumor volume, compared with a control. The bodyweights of mice inoculated with *INHBA*-overexpressed cells decreased dramatically, and these mice all died at an early stage, suggesting the occurrence of activin-induced cachexia. Our findings indicated that the activin signal can promote cancer progression in a subset of PC and might be involved in cachexia. The activin signal might be a novel target for the treatment of PC.

Introduction

Pancreatic cancer (PC) is a devastating disease. Gemcitabine has been the standard therapy in patients with advanced PC for over a decade, but recently, the overall survival (OS) has been significantly prolonged using combination therapies, such as gemcitabine plus erlotinib, a combination of oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLFIRINOX), or a combination of nab-paclitaxel and gemcitabine [1–3]. Despite some recent progress, however, the OS rate of patients with PC is still less than 5% [4].

The progression of PC is thought to be influenced by multiple genetic alterations. During early genetic events, such as activating mutations in the *K-ras* oncogene, pancreatic duct lesions show minimal cytological and architectural atypia. The inactivation of the *p16* tumor suppressor gene appears to occur at a later stage, followed by the loss of the *p53*, *SMAD4*, and *BRCA2* tumor suppressor genes [5]. Despite such recent breakthroughs in the molecular biology of PC, the inhibition of the epidermal growth factor receptor using erlotinib is, to date, the only targeted approach that has demonstrated a survival benefit [1]. Therefore, further understanding of the molecular biology of PC is needed.

The transforming growth factor, beta (TGFB) receptor II (*TGFBR2*) and *SMAD4* genes are commonly inactivated in several types of cancer, providing evidence that the TGFB signal functions as a tumor suppressor [6,7]. The loss of the *SMAD4* gene eliminates the classic SMAD2/3/4 heteromeric complexes that have been implicated in a large number of TGFB-dependent transcriptional regulatory complexes. As a result, TGFB-mediated growth inhibition is lost. The *SMAD4* gene is inactivated in 55% of PC tumors, and numerous studies on the TGFB signal in PC have been reported [5,8]. In addition, pancreatic-specific *TGFBR2* or *SMAD4*-knockout mice with active *K-ras* expression reportedly developed PC [9,10]. However, few studies regarding the activin signal, which also belongs to the TGFB superfamily, have been reported [11–13]. Activins are related dimeric proteins and mature activins are composed of two inhibin beta subunits (beta A and beta B encoded by the *INHBA* and *INHBB* genes, respectively). These subunits assemble into the active dimeric growth factors activin A (beta A, beta A), activin B (beta B, beta B), and activin AB (beta A, beta B) [14]. Defects in several genes involved in the activin signal pathway have been characterized in several cancers, and the activin signal induces growth inhibition and apoptosis mainly through SMAD-dependent pathways [15–21]. In contrast, however, a recent study has demonstrated that the activin

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signal is associated with self-renewal and the tumorigenicity of PC stem cells [13], and several articles have demonstrated that upregulated *INHBA* expression may promote cancer cell proliferation in several cancers [22–24]; thus, the role of the activin signal in pancreatic carcinogenesis remains controversial.

In our previous study, we identified the homozygous deletion of the activin A receptor, type IB (*ACVR1B*) gene in PC cell lines and clinical samples and showed that the activin signal has a tumor suppressive role [21]. In the present study, we analyzed the association between the plasma activin A levels and survival in patients with advanced PC, but the results were contrary to our expectations. Thus, we investigated the roles of the activin signal, other than tumor suppression, in PC.

Materials and methods

Patients and sample collection

A total of 38 patients with a good performance status (PS) who had been diagnosed as having unresectable PC based on the results of an endoscopic biopsy performed at Kindai University Hospital between April 2007 and March 2008 were enrolled. This study was retrospectively performed and was approved by the institutional review board of the Kindai University Faculty of Medicine. Among those who received chemotherapy at Kindai University Hospital, the progression-free survival (PFS) was defined as the time from the initiation of chemotherapy until the first observation of disease progression or death from any cause, while OS was defined as the time from the initiation of chemotherapy until death from any cause. The response to chemotherapy was evaluated at 1 month after the start of therapy and every 2 months thereafter using computed tomography according to the Response Evaluation Criteria in Solid Tumors.

Blood samples were collected at the time of diagnosis. Peripheral venous blood was drawn into a tube containing ethylenediaminetetraacetic acid (EDTA) and was immediately prepared by centrifugation at 1200 × *g* for 10 min. The plasma samples were stored at −80 °C until use.

ELISA for activin A

The plasma concentrations and cell culture medium for activin A were determined using a specific sandwich ELISA according to the manufacturer's instructions (R&D Systems; Minneapolis, MN), as previously described [25].

Cell culture, ligands, and reagents

Sui65, Sui66, Sui67, Sui68, Sui69, Sui70, Sui71, Sui73, Sui74, and MIA PaCa-2 cell lines (human PC cell lines) were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) [26]. The cell lines were maintained in a 5% CO_2-humidified atmosphere at 37 °C.

Activin A and follistatin (an antagonist of activin) were purchased from R&D Systems. The ACVR1B/TGFBR1/ACVR1C-specific inhibitor SB431542, the AKT-specific inhibitor LY294002, the JNK-specific inhibitor SP600125, 5-FU, and gemcitabine were purchased from Sigma-Aldrich.

Plasmid construction, viral production, and stable transfectants

The methods used in this section have been previously described [25]. Briefly, the cDNA fragment encoding the human full-length *INHBA* gene was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa, Otsu, Japan). A full-length cDNA fragment was introduced into a pQCLIN retroviral vector (Clontech; Palo Alto, CA) together with enhanced green fluorescent protein (EGFP) following the internal ribosome entry site sequence (IRES) to monitor the expression of the inserts indirectly. The vectors and the stable viral transfectant MIA PaCa-2 and Sui66 cell lines were designated as pQCLIN-EGFP, pQCLIN-INHBA, MIA PaCa/EGFP, MIA PaCa/INHBA, Sui66/EGFP, and Sui66/INHBA, respectively.

Real-time reverse-transcription PCR (RT-PCR)

One microgram of total RNA from the cultured cell lines and normal pancreas tissue purchased from Clontech were converted to cDNA using the GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using SYBR Premix Ex Taq and Thermal Cycler Dice (TaKaRa), as described previously [25]. The experiment was performed in triplicate.

Western blot analysis

A western blot analysis was performed as described previously [25]. When the influence of the ligands was evaluated, the cultured medium was replaced with 1% FBS medium 8 hours before exposure to the ligands. Inhibitors were added three hours before sample collection. Rabbit antibodies specific for SMAD2, phospho-SMAD2, AKT, phospho-AKT, ERK, phosphor-ERK, JNK, phospho-JNK, p21, and β-actin were obtained from Cell Signaling (Beverly, MA).

Cellular growth and inhibitory assay

The cellular growth and growth-inhibitory effects of 5-FU and gemcitabine in the transfectant cell lines were examined using a 3, 4, 5-dimethyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) assay, as described previously [25,27]. To evaluate growth in the presence of ligands and inhibitors, we used 1% FBS medium [21]. The experiment was performed in triplicate.

Colony formation assay

The transfectant cells (MIA PaCa-2 and Sui66) were seeded into a six-well plate and a colony formation assay was performed, as previously described [21]. The experiment was performed in triplicate.

Xenograft studies

Nude mice (BALB/c nu/nu; 6-week-old females; CLEA Japan, Inc.) were used for the *in vivo* studies, described previously [25], and were cared for in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kindai University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines.

Statistical analysis

Continuous variables were analyzed using the Student *t*-test, and the results were expressed as the average and standard deviations (SD). The univariate relationship between each independent variable was examined using the χ^2 test or the Fisher exact test if there were five or fewer observations in a group. The PFS and OS were analyzed using the Kaplan–Meier method and were compared among groups using the log-rank test. The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft, Redmond, WA). A *P* value of less than 0.05 was considered statistically significant.

Results

Plasma activin A levels and patient characteristics

A total of 38 patients with advanced PC and a good PS (0 or 1) were enrolled in this study (Table 1). The patients with PC ranged in age from 54 to 82 years, with a median of 68 years, and the male:female ratio was 22:16. The plasma activin A concentrations determined using an ELISA assay ranged from 266.7 to 2711 pg/mL, with a mean of 827.8 ± 483.8 pg/mL and a median of 667.5 pg/mL. When the cut-off was set at 800 pg/mL, no significant difference in the patient characteristics was observed between the low and high groups, except for the incidence of distant metastasis (Table 1).

Prognostic significance of plasma activin A in patients with advanced PC

Twenty-eight patients received chemotherapy (gemcitabine, n = 17; gemcitabine/S1, n = 7; S1, n = 4) at Kindai University Hospital. These regimens were commonly used in Japan before the availability of erlotinib, FOLFIRINOX, or nab-paclitaxel. Among these patients, no significant difference was observed in the response rate to chemotherapy between the low and high plasma activin A groups (8/17 vs. 6/11, $P = 0.70$) (Table 1). In contrast, patients with high plasma activin A levels tended to have a shorter PFS than those with low levels, although the difference was not significant (median PFS; 240 vs. 312 days, $P = 0.083$) (Fig. 1a). Furthermore, patients with high levels had a significantly shorter OS than those with low levels (median OS; 345 vs. 438 days, $P = 0.034^*$) (Fig. 1b). Patients with distant metastasis had significantly higher activin A concentrations than those without (1004.21 ± 595.28 vs. 651.41 ± 241.76 pg/mL, $P = 0.022^*$) (Fig. 1c). Then, distant metastasis was also analyzed,

Table 1
Patient characteristics and associations with plasma activin A levels.

Patients characteristics	Plasma activin A		*P*-values
	<800 pg/mL (n = 23)	≥800 pg/mL (n = 15)	
Age			
<70 years	15	10	0.93
≥70 years	8	5	
Gender			
Male	14	8	0.65
Female	9	7	
Lymph node metastasis			
Negative	5	2	0.68
Positive	18	13	
Distant metastasis			
Negative	15	4	0.020*
Positive	8	11	
Treatment			
Best supportive care	3	3	0.67
Chemotherapy	17	11	
Unknown	3	1	
Response to chemotherapy			
PR	8	6	0.70
SD or PD	9	5	
Median PFS (days)	312	240	0.083
Median OS (days)	482	342	0.034*

PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival.
PFS and OS were analyzed using the log-rank test, and the others were analyzed using the χ^2 test or the Fisher exact test if there were five or fewer observations in a group.
* $P < 0.05$.

and no significant difference in the OS was seen (median OS: 345 vs. 438 days, $P = 0.13$) (Fig. 1d). These findings suggest that plasma activin A can be a predictive factor for tumor progression and survival in patients with advanced PC.

INHBA and INHBB mRNA expressions and the influence of follistatin and activin A in several PC cell lines

Considering the results of several previous studies, including our study showing the tumor suppressive role of the activin signal, the negative prognostic significance of the plasma activin A level was contrary to our expectations. Consequently, the mRNA expressions of *INHBA* and *INHBB* (inhibin, beta subunits) were evaluated in normal pancreatic tissue, the hTERT-HPNE cell line (a normal pancreatic duct cell line), and several PC cell lines using real time RT-PCR. The Sui71 and Sui74 cell lines exhibited a high expression level of *INHBA* or *INHBB* mRNA (Fig. 2a).

Next, to investigate the role of highly expressed *INHBA* and *INHBB*, the effects of follistatin, an antagonist of activin, on the proliferations of the Sui71 and Sui74 (high expression of *INHBA* or *INHBB* mRNA) and the Sui66 and MIA PaCa-2 (low expressions) cell lines were investigated using an MTT assay. The cellular growth of the Sui71 and Sui74 cell lines (high expressions) was inhibited using follistatin, whereas that of the Sui66 and MIA PaCa-2 cell lines (low expressions) was not changed (Fig. 2b and c). In our previous study, activin A inhibited the cellular growth in the Sui66 and Sui73 cell

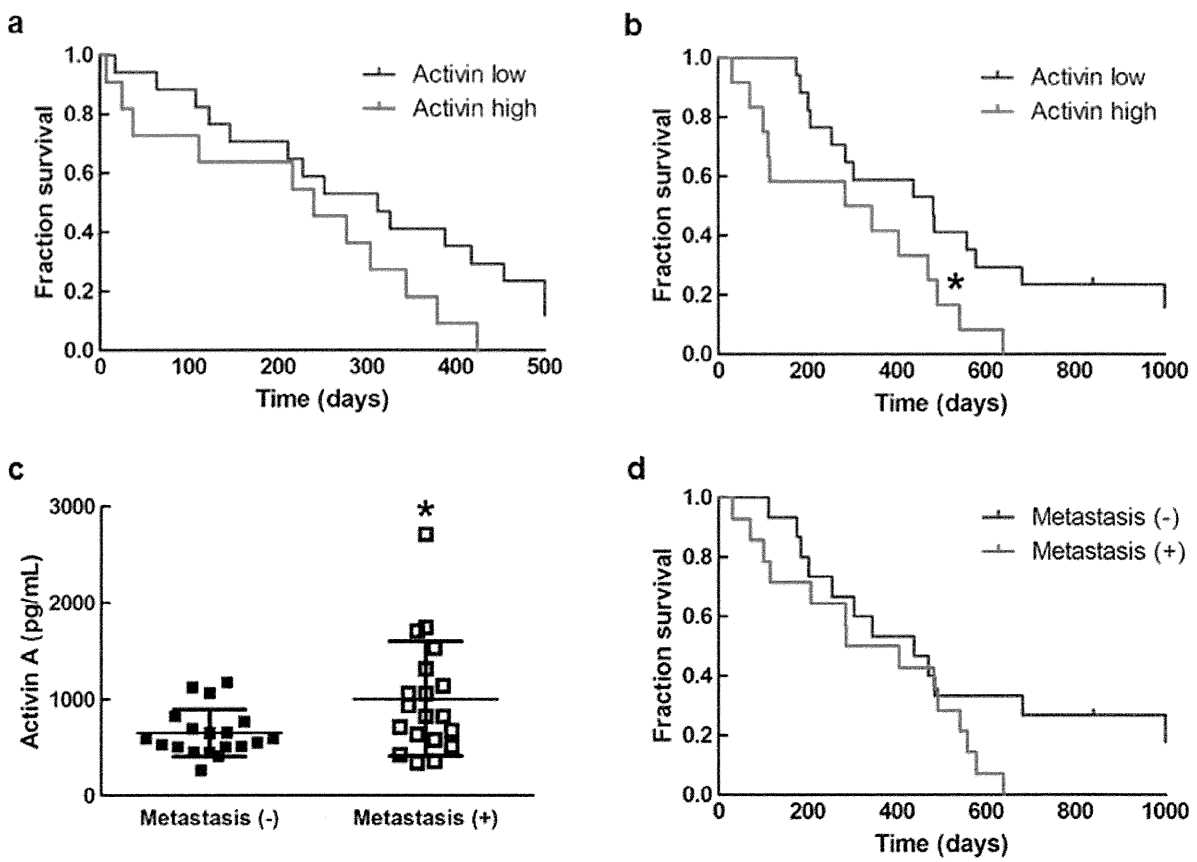

Fig. 1. Plasma activin A levels and prognosis. (a) Kaplan–Meier curve of PFS between patients with high plasma activin A levels and those with low levels. Patients with high plasma activin A levels tended to have a shorter PFS (although the difference was not significant) than those with low levels (median PFS; 240 vs. 312 days, $P = 0.083$). (b) Kaplan–Meier curve of OS between patients with high plasma activin A and those with low levels. Patients with high levels had a significantly shorter OS than those with low levels (median OS; 345 vs. 438 days, $P = 0.034$*). (c) Association between distant metastasis and plasma activin A concentrations. Patients with distant metastasis had significantly higher activin A concentrations than those without (1004.21 ± 595.28 vs. 651.41 ± 241.76 pg/mL, $P = 0.022$*). (d) Kaplan–Meier curve of OS between patients with distant metastases and those without distant metastasis. No significant difference in OS was observed (median OS; 345 vs. 438 days, $P = 0.13$).

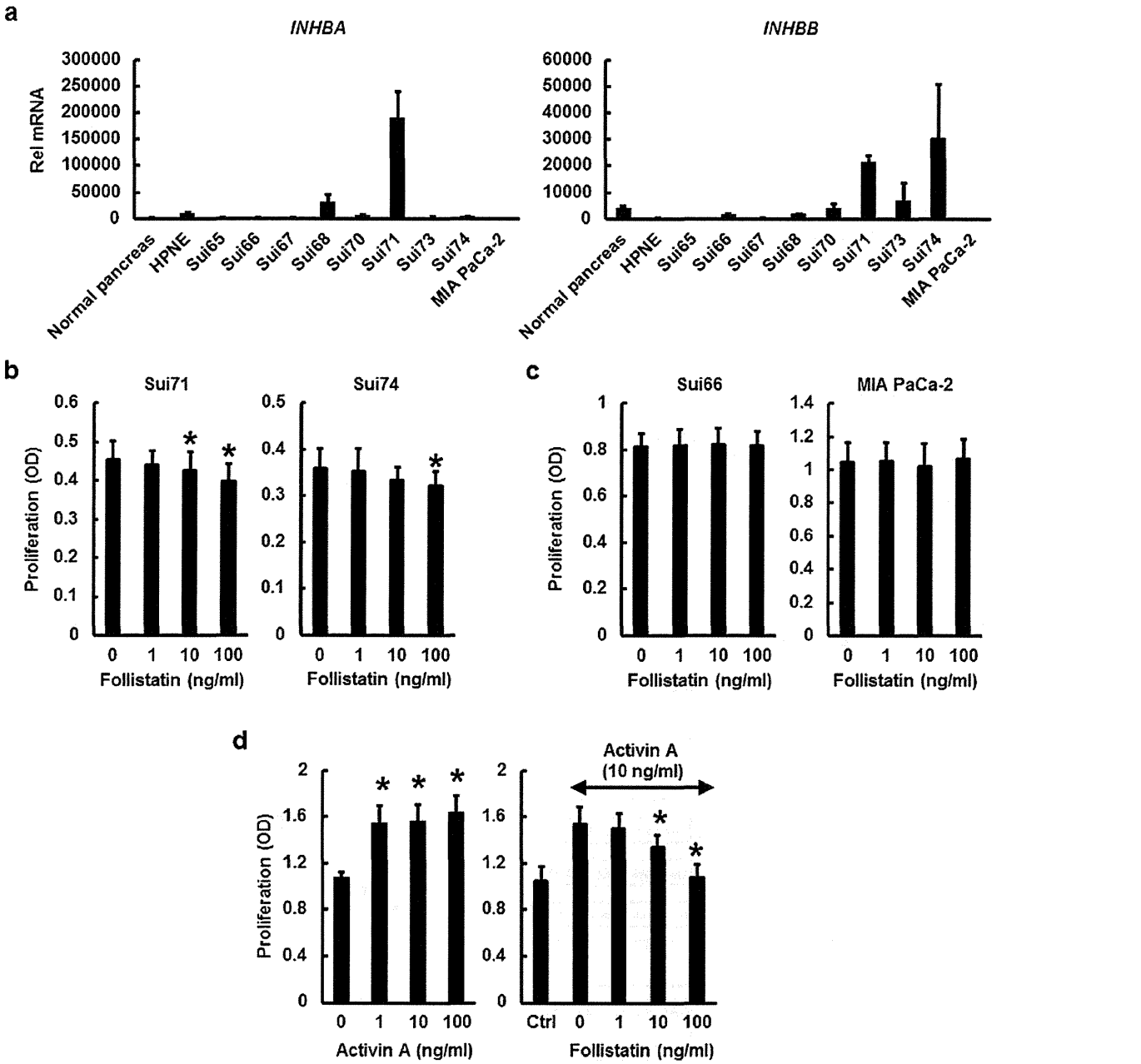

Fig. 2. Expressions of *INHBA* and *INHBB* mRNA in PC cell lines and the effect of activin signals on the proliferation of the PC cell lines. Real-time RT-PCR was used to evaluate the *INHBA* and *INHBB* mRNA expression levels in normal pancreatic tissue, the hTERT-HPNE cell line (normal pancreatic duct cell line) and several PC cell lines. To evaluate growth in the presence of ligands, we used an MTT assay with 1% FBS medium. The experiments were performed in triplicate. (a) *INHBA* and *INHBB* mRNA expression levels. The Sui71 and Sui74 cell lines had high expression levels of *INHBA* or *INHBB* mRNA. Columns, mean of independent triplicate experiments; Bars, SD; Rel mRNA, *INHBA* or *INHBB*/*GAPD*×10^6. (b) Influence of follistatin on cellular growth in cell lines with high *INHBA* or *INHBB* mRNA expression levels. The cellular growth of the Sui71 and Sui74 cell lines was inhibited by follistatin (1 ng/mL, $P = 0.27$ and 0.32; 10 ng/mL, $P = 0.011^*$ and 0.12; 100 ng/mL, $P = 0.018^*$ and 0.039*, respectively). Columns, mean of independent triplicate experiments; Bars, SD; $^*P < 0.05$. (c) Influence of follistatin on cellular growth with low *INHBA* and *INHBB* mRNA expression levels. The cellular growth of the Sui66 and MIA PaCa-2 cell line was not changed (1 ng/mL, $P = 0.87$ and 0.70; 10 ng/mL, $P = 0.73$ and 0.54; 100 ng/mL, $P = 0.86$ and 0.37, respectively). Columns, mean of independent triplicate experiments; Bars, SD; $^*P < 0.05$. (d) Influence of activin A on the cellular growth of the MIA PaCa-2 cell line. Activin A greatly enhanced the cellular growth (1 ng/mL, $P = 0.045^*$; 10 ng/mL, $P = 0.043^*$; 100 ng/mL, $P = 0.032^*$), and the enhanced cellular growth (activin A, 10 ng/mL) was cancelled by follistatin (1 ng/mL, $P = 0.24$; 10 ng/mL, $P = 0.022^*$; 100 ng/mL, $P = 0.0057^*$). Ctrl, control (no ligand); Columns, mean of independent triplicate experiments; Bars, SD; $^*P < 0.05$.

lines (wild-type *ACVR1B* and *SMAD4* gene) [21]. In the MIA PaCa-2 cell line (wild-type *ACVR1B* and *SMAD4* gene), however, activin A greatly enhanced the proliferation, which was cancelled by follistatin (Fig. 2d). These findings suggest that activin A enhances cellular growth in a subset of PC.

Non-SMAD pathways were activated by activin A and the inhibitors cancelled the enhanced proliferation in the MIA PaCa-2 cell line

The SMAD pathway is known as a tumor suppressor, inhibiting cellular growth in several cancers, whereas non-SMAD pathways,

including MAPK, PI3K/AKT, and so on, enhance cellular growth [6,7,28]. Therefore, non-SMAD pathways were investigated to address the mechanism responsible for the cellular growth enhanced by activin A. The MIA PaCa-2 cell line, the cellular growth of which was enhanced by activin A, and the Sui66 cell line, the cellular growth of which was inhibited by activin A, were used at an activin A concentration of 10 ng/mL. Activin A increased the phosphorylation of SMAD2 in both the MIA PaCa-2 and Sui66 cell lines, whereas it increased the phosphorylation of AKT and JNK in the MIA PaCa-2 cell line but not in the Sui66 cell line (Fig. 3a). The phosphorylation of ERK was elevated before activin A stimulation and was not changed after activin A stimulation (Fig. 3a). Next, we evaluated the expression levels of $p21^{CIP1/WAF1}$. $p21^{CIP1/WAF1}$ is a major cdk inhibitor and is a hallmark of the cytostatic role of the TGFB signal pathway [29]. The expression of p21 was increased by activin A in the Sui66 cell line. In the MIA PaCa-2 cell line, however, its expression was reduced by activin A (Fig. 3a). In addition, an ACVR1B inhibitor (SB431452; 10 μM), an AKT inhibitor (LY294002; 10 μM), and a JNK inhibitor (SP600125; 20 μM) inhibited the cellular growth of the MIA PaCa-2 cell line enhanced by activin A (Fig. 3b). Therefore, we speculated that the cellular growth of the MIA PaCa-2 cell line may be enhanced by activin A via a non-SMAD pathway.

INHBA-overexpression enhanced cellular growth and colony formation in the MIA PaCa-2 cell line

To investigate the influence of a high *INHBA* mRNA expression level, *INHBA*-overexpressed cell lines were created using the retroviral method. Overexpression of the *INHBA* gene was confirmed using an ELISA and western blot analyses for the phosphorylation of SMAD2 (Fig. 4a). *INHBA*-overexpression enhanced cellular growth and colony formation in the MIA PaCa-2 cell line (Fig. 4b and c). However, the sensitivities to 5-FU and gemcitabine were not changed by *INHBA*-overexpression (50% inhibitory concentration [IC_{50}] of 5-FU; EGFP, 3.36 μM vs. INHBA, 3.62 μM and IC_{50} of gemcitabine; EGFP, 0.28 μM vs. INHBA, 0.29 μM, respectively). These findings suggest that a high *INHBA* mRNA expression level enhances cellular growth in a subset of PC but is not associated with sensitivity to chemotherapy. In the Sui66 cell line, however, cellular growth and colony formation were not enhanced instead slightly (but not significantly) suppressed by *INHBA*-overexpression (Fig. S1), which was reasonable since the cellular growth of the Sui66 cell line was inhibited by activin A via the SMAD pathway [21]. Thus, another subset of PC in which cellular growth is not enhanced by a high *INHBA* mRNA expression level seems to exist, and this feature was seen in the Sui66 cell line.

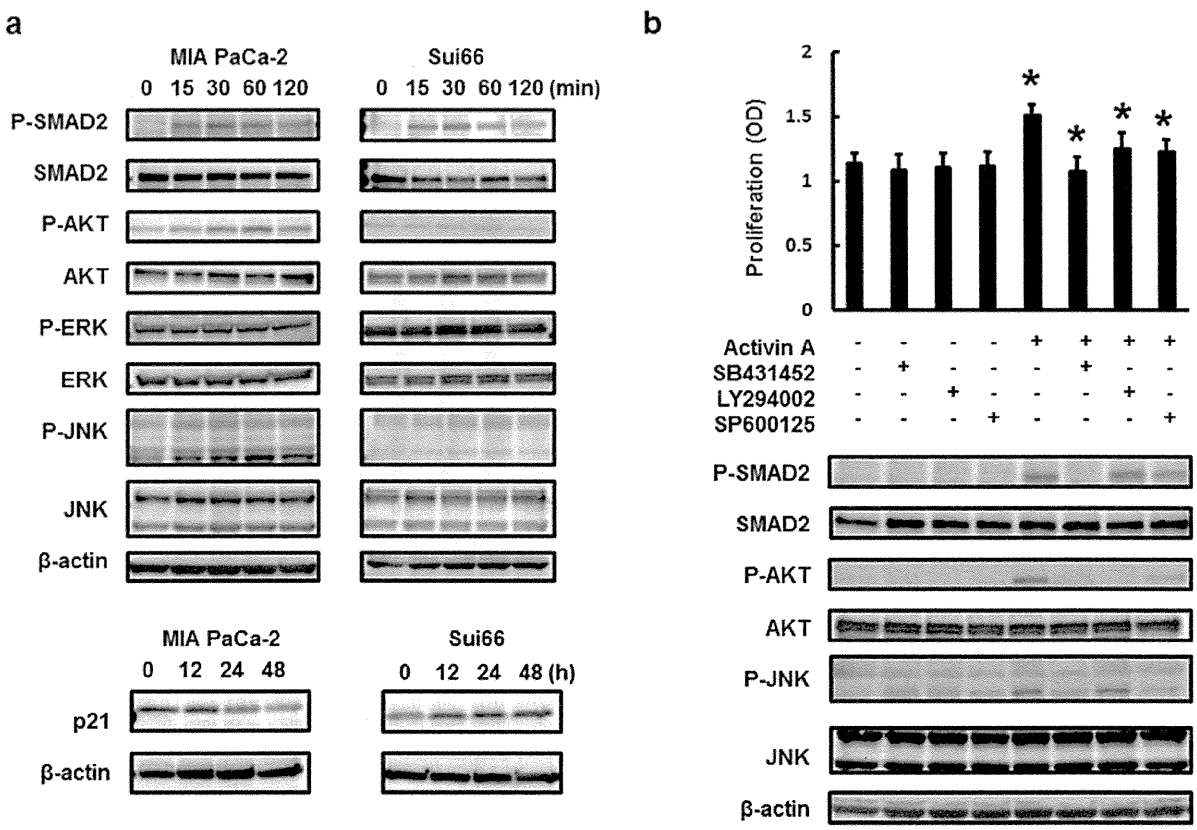

Fig. 3. Western blot analyses and growth inhibition of inhibitors. (a) Western blot analyses of SMAD and non-SMAD pathway components in MIA PaCa-2 and Sui66 cell lines. Activin A (10 ng/mL) increased the phosphorylation of SMAD2, AKT, and JNK in the MIA PaCa-2 cell line, the cellular growth of which was enhanced by activin A. In contrast, activin A increased the phosphorylation of SMAD2 but not of AKT or JNK in the Sui66 cell line, the cellular growth of which was inhibited by activin A. The phosphorylation of ERK was elevated before activin A stimulation and was not changed after activin A stimulation. The expression of p21 was increased by activin A in the Sui66 cell line. In the MIA PaCa-2 cell line, however, its expression was reduced by activin A. β-actin was used as an internal control. (b) Growth inhibition of inhibitors. To evaluate growth in the presence of ligands and inhibitors, we used an MTT assay with 1% FBS medium. The experiment was performed in triplicate. The ACVR1B inhibitor (SB431452, 10 μM), the AKT inhibitor (LY294002, 10 μM), and the JNK inhibitor (SP600125, 20 μM) did not inhibit the cellular growth of MIA PaCa-2 without activin A ($P = 0.28$, 0.31, 0.41, respectively). In contrast, the cellular growth enhanced by 10 ng/mL of activin A ($P = 0.012$*) was inhibited by the inhibitors ($P = 0.0070$*, 0.030*, 0.031*, respectively). β-actin was used as an internal control. Columns, mean of independent triplicate experiments; Bars, SD; *$P < 0.05$.

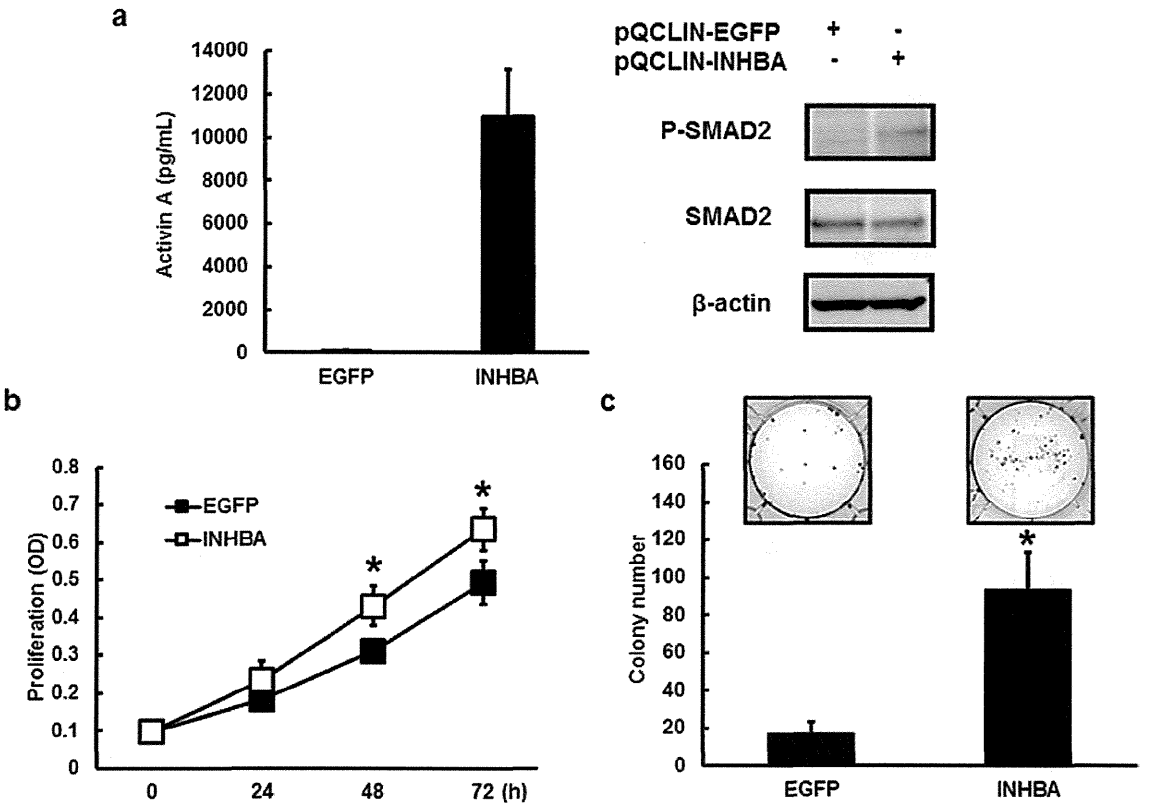

Fig. 4. Influence of *INHBA*-overexpression in the MIA PaCa-2 cell line. To investigate the influence of high *INHBA* mRNA expression levels, an *INHBA*-overexpressed MIA PaCa-2 cell line was created using a retroviral method. (a) ELISA for activin A and western blot analyses. The level of activin A in cell cultured medium of the MIA PaCa/INHBA cell line was elevated compared with the control (10984.3 ± 2212.1 pg/mL). SMAD2 was also phosphorylated in the MIA PaCa/INHBA cell line. β-actin was used as an internal control. Columns, mean of independent triplicate experiments; Bars, SD. (b) Cellular growth. To evaluate cellular growth, we used an MTT assay with 1% FBS; the experiment was performed in triplicate. *INHBA*-overexpression enhanced the cellular growth (0 h, $P = 0.89$; 24 h, $P = 0.10$; 48 h, $P = 0.013$*; 72 h, $P = 0.022$*). Lines, mean of independent triplicate experiments; Bars, SD; *$P < 0.05$. (c) Colony formation. MIA PaCa-2 transfectant cells were seeded into a six-well plate of 1000 cells/well with 1% FBS, and the colonies were counted after 2 weeks. The experiment was performed in triplicate. Colony formation was enhanced by *INHBA*-overexpression (EGFP, 17.67 ± 5.90 vs. INHBA, 94 ± 19.67, $P = 0.010$*). Column, mean of independent triplicate experiments; Bars, SD; *$P < 0.05$.

Xenograft study

We evaluated the *in vivo* tumor growth of MIA PaCa-2 transfectant cell lines. MIA PaCa/INHBA cells tended to exhibit a large tumor volume, compared with control cells (EGFP, 349.7 ± 149.7 mm^3 vs. INHBA, 482.4 ± 182.4 mm^3; $P = 0.24$ on day 22; and EGFP, 447.9 ± 195.0 mm^3 vs. INHBA, 549.9 ± 257.7 mm^3; $P = 0.50$ on day 26, respectively) (Fig. 5a). After day 29, however, the MIA PaCa/INHBA-inoculated mice died one after another, resulting in a significantly shorter survival period than in the control ($P = 0.0019$*) (Fig. 5b). The bodyweights of the MIA PaCa/INHBA-inoculated mice decreased by the day and became significantly lower than those of the MIA PaCa/EGFP-inoculated mice (EGFP, 22.3 ± 0.96 g vs. INHBA, 18.3 ± 2.94 g; $P = 0.020$* on day 22; and EGFP, 23.2 ± 1.26 g vs. INHBA, 15.9 ± 3.8 g; $P = 0.0036$* on day 26, respectively) (Fig. 5c and d). An autopsy demonstrated that there was no distant metastasis. The heart muscles of MIA PaCa/INHBA-inoculated mice were clearly atrophied, compared with those of the control (Fig. 5d). Several previous studies have shown that the activin signal is related to cachexia [30–33], and our *in vivo* experimental findings were consistent with these previous studies.

Discussion

Activins are related dimeric proteins that belong to the TGFB superfamily of growth and differentiation factors [14]. Several previous studies, including our study, have shown an anti-tumorigenic effect of the activin signal in many cancers. In these studies, activin A induces growth inhibition and apoptosis mainly through SMAD-dependent pathways [15–21]. Our present study revealed similar results in the Sui66 cell line. In contrast, however, several studies have demonstrated that the activin signal promotes cancer progression [22–24]. In this present study, several PC cell lines had high *INHBA* and *INHBB* mRNA expression levels, and the cellular growth of these cell lines was inhibited by the activin antagonist follistatin. Furthermore, in contrast to the Sui66 cell line (low expressions of *INHBA* and *INHBB*), the MIA PaCa-2 cell line (low expressions of *INHBA* and *INHBB*) was greatly enhanced by activin A via a non-SMAD pathway, and this enhancement was cancelled by the antagonist follistatin. That is, our present study indicated that the activin signal promotes cancer progression in a subset of PC. Furthermore, the plasma activin A level can be a novel prognostic factor in patients with advanced PC. To the best of our knowledge, this is the first study to show the role of the activin signal in cancer progression role via a non-SMAD pathway and the prognostic significance of the plasma activin A level in PC.

Non-SMAD pathways include MAPK signals, PI3K/AKT signals, and so on [28]. The ERK signal, which belongs to a MAPK family that enhances cellular proliferation, was activated before activin A stimulation, probably because of a *K-RAS* mutation. Otherwise, in the MIA PaCa-2 cell line, the JNK signal (another MAPK signal) and PI3K/AKT signal were activated by the stimulation, the inhibition of which

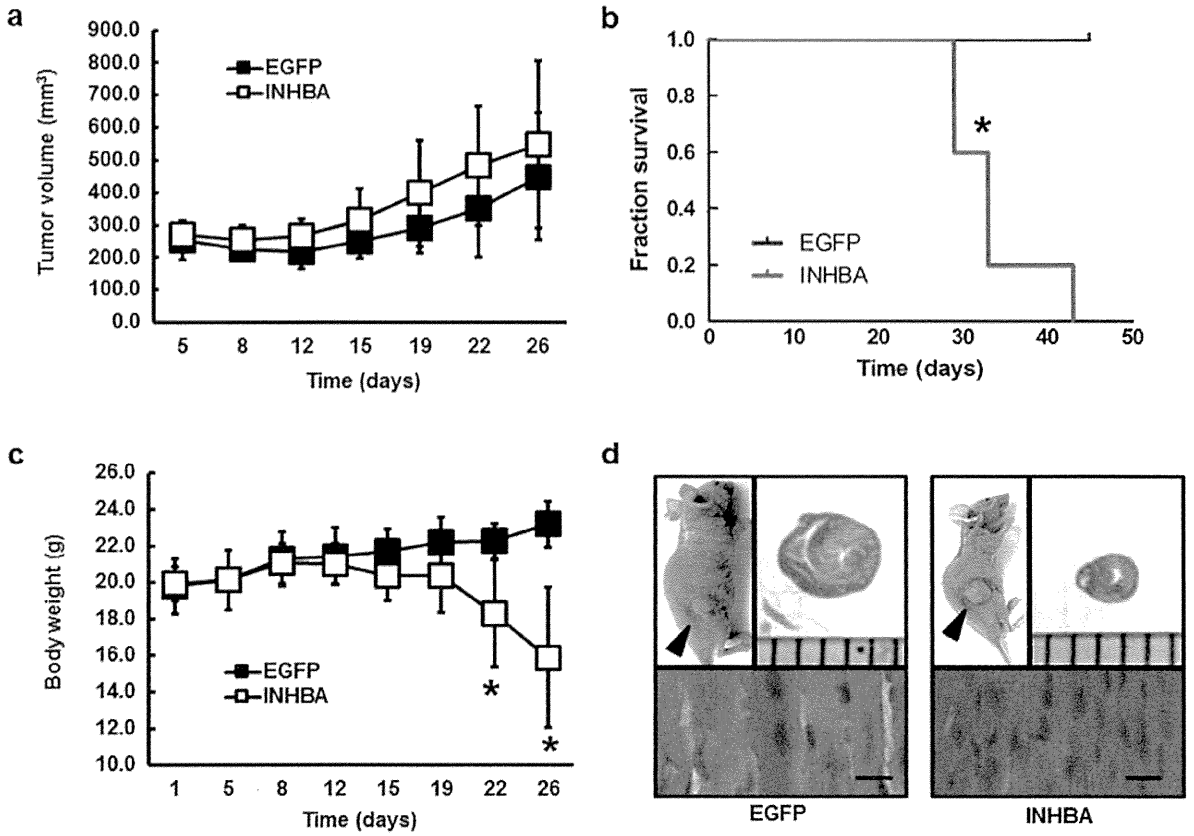

Fig. 5. Xenograft study. To evaluate the *in vivo* tumor growth of MIA PaCa-2 transfectant cell lines, a suspension of 5×10^6 MIA PaCa-2 transfectant cells (in 50 μL PBS) with 50 μL of Matrigel was subcutaneously inoculated into the right flank of nude mice (n = 5). (a) Tumor volume. MIA PaCa/INHBA cells tended to produce a large tumor volume, compared with the control cells (EGFP, 349.7 ± 149.7 mm^3 vs. INHBA, 482.4 ± 182.4 mm^3; $P = 0.24$ on day 22; and EGFP, 447.9 ± 195.0 mm^3 vs. INHBA, 549.9 ± 257.7 mm^3; $P = 0.50$ on day 26, respectively). Lines, mean of tumor volume (n = 5); Bars, SD. (b) Kaplan–Meier curve of mice. After day 29, the MIA PaCa/INHBA-inoculated mice died one after another, resulting in a significantly shorter survival period than the control group ($P = 0.0019^*$). $^*P < 0.05$. (c) Bodyweight. The bodyweights of MIA PaCa/INHBA-inoculated mice decreased daily and became significantly lower than those of MIA PaCa/EGFP-inoculated mice (EGFP, 22.3 ± 0.96 g vs. INHBA, 18.3 ± 2.94 g; $P = 0.020^*$ on day 22; and EGFP, 23.2 ± 1.26 g vs. INHBA, 15.9 ± 3.8 g; $P = 0.0036^*$ on day 26, respectively). Lines, mean of bodyweight (n = 5); Bars, SD, $^*P < 0.05$. (d) Photographs of mice sacrificed on day 43. The MIA PaCa/INHBA-inoculated mouse appeared to be scrawny, compared with the control, and its heart size (HE staining; cross section) was also clearly smaller than that of the control. The heart muscle fibers of the MIA PaCa/INHBA-inoculated mouse were also clearly atrophied, compared with those of the control (HE staining; ×200). Ladder, 1 mm; Scale bar, 20 μm.

cancelled the cellular growth enhanced by activin A. In the Sui66 cell line, however, the non-SMAD pathway was not activated, but the SMAD pathway was activated by activin A, resulting in the inhibition of cellular growth. These findings suggest that activin signal exerts a tumor progressive role via a non-SMAD pathway in a subset of PC and that this signal has a tumor suppressive role via the SMAD pathway in another subset of PC. The detailed mechanism responsible for this difference remains unclear, and further research should be performed.

JNK is a member of the MAPK family, and controls cell proliferation, differentiation, apoptosis, and survival [34,35]. The JNK signal reportedly plays an important role in the development of various cancers. The activation of JNK has been reported in human PC specimens [36], and the growth of PC is reportedly inhibited by the pharmacological inhibition of JNK [37–39]. The family of lipid kinases known as PI3K are regarded as key regulators in many essential cellular processes, including cell survival, growth, and differentiation [40–42]. The AKT-mediated activation of downstream targets, including mammalian target of rapamycin, stimulates cell proliferation and the regulation of translation in response to growth factors by the phosphorylation of the protein synthesis machinery [40–42]. Reportedly, the PI3K/AKT signal is activated and AKT2 is amplified in a considerable number of pancreatic cancer cases [43–45]. Thus, these processes might be involved in the activin A-induced enhancement of cellular growth in PC.

In our *in vivo* study, MIA PaCa/INHBA cells tended to produce a larger tumor volume than the control cells, but no significant difference was demonstrated, possibly because the mice died before any difference could be observed. In other words, the bodyweights of the MIA PaCa/INHBA-inoculated mice decreased daily as a result of cachexia, and the mice had a significantly shorter survival period than the control mice. Indeed, as noted, an association between activin A and cachexia, or muscle loss, has been reported in several recent studies [30–33]. Although the mechanism by which activin A promotes cachexia has not been fully elucidated, previous studies have suggested that there may be multiple pathways that impinge by both inhibiting TORC1 pathways that promote protein synthesis and by the activation of SMAD2/3, which mediates the inhibition of genes associated with muscle differentiation [46–48]. Similarly, a recent study has shown that activin A induces muscle wasting by activating SMAD2/3 transcription factors, which increase protein degradation by up-regulating the expression of the muscle-specific ubiquitin ligase atrogin-1 and depress protein synthesis via the inhibition of the AKT/TORC pathway [33].

Several previous studies have shown the prognostic significance of the expression of *INHBA* in several cancers, but the plasma

activin A level has not been analyzed [22–24]. In our clinical data, although the number was small, patients with high plasma activin A levels tended to have a shorter PFS (although the difference was not significant) and a significantly shorter OS than those with low levels, despite no significant difference in the response to chemotherapy. In the experimental study, no significant difference in the sensitivities to 5-FU and gemcitabine was observed between the MIA PaCa/EGFP and MIA PaCa/INHBA cell lines. These findings suggest that the plasma activin A level might not be associated with the response to chemotherapy, but with cancer progression and survival in patients with advanced PC. In addition, considering the bodyweight loss and early death of MIA PaCa/INHBA-inoculated mice, the poor prognosis of the patients with high plasma activin A levels might have been caused by not only cancer progression, but also by cachexia, although this possibility could not be further investigated because of a lack of clinical data. Interestingly, similar to previous studies [49], patients with distant metastasis had higher plasma activin A concentrations than those without distant metastasis. No significant difference was observed in the OS between patients with distant metastasis and those without distant metastasis in our clinical data; therefore, the plasma activin A level, which was associated with distant metastasis, seems to be a stronger prognostic factor than distant metastasis.

In conclusion, we found that several PC cell lines had high *INHBA* or *INHBB* mRNA expression levels and that the activin signal enhanced cellular growth in a subset of PC via a non-SMAD pathway. Since these findings are contradictory to its tumor suppressive role reported by several previous studies, including ours, further research should be performed. In addition, activin A is associated with cachexia. Based on these roles of activin signals, the plasma activin A level might be a predictor of a poor prognosis among patients with advanced PC. These findings indicate that the activin signal might be a novel target for the treatment of PC.

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Conflict of interest

The authors have no conflict of interest in this work.

Appendix: Supplementary material

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Performance of a novel *KRAS* mutation assay for formalin-fixed paraffin embedded tissues of colorectal cancer

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Abstract

We compared the performance of the 3D-Gene® mutation assay (3D-Gene® *KRAS* mutation assay kit) with the Scorpion-ARMS (therascreen® KRAS RGQ PCR Kit) and Luminex (MEBGEN™ KRAS kit) assays for the detection of *KRAS* mutations in formalin-fixed, paraffin-embedded tissue samples from 150 patients diagnosed with colorectal cancer. DNA was extracted from the paraffin-embedded tissue samples with or without macrodissection under hematoxylin and eosin staining and the *KRAS* mutation status was independently determined using these assays. Discordant results were re-analyzed by Sanger sequencing. Mutation detection analysis was successfully performed in all 150 specimens using the 3D-Gene® mutation assay without an invalid case. The concordance rate between the 3D-Gene® mutation assay and Scorpion-ARMS or Luminex was 98.7% (148/150). *KRAS* mutations were detected at a frequency of 35.3% (53/150) in colorectal cancer specimens. Three discrepant cases were found between the three assays. Overall, our results demonstrate a high concordance rate of between the 3D-Gene® mutation assay and the two existing *in-vitro* diagnostics kits. All three assays proved to be validated methods for detecting clinically significant *KRAS* mutations in paraffin-embedded tissue samples.

Keywords: Colorectal cancer; *KRAS* mutation; Anti-EGFR antibody; 3D-Gene® *KRAS* mutation assay; Companion diagnosis

Background

Several studies have shown that cetuximab and panitumumab do not improve progression-free or overall survival for advanced colorectal cancer patients with mutated *KRAS* (Karapetis et al. 2008; Amado et al. 2008; De Roock et al. 2008; Lièvre et al. 2006, 2008). KRAS is a GTP binding protein with a molecular weight of 21 kDa. KRAS is activated by GTP binding triggered by upstream signals such as EGFR. The *KRAS* gene is located on chromosome 12 and consists of 4 exons and 3 introns. Single base mutations of *KRAS* genes decrease GTPase activity and leads to constitutive activation of KRAS. The frequency of *KRAS* mutations is 34.6% in colorectal cancer, according to the COSMIC (Catalogue of Somatic Mutation in Cancer) database. Comparable frequencies have also been reported in population–based studies. (Andreyev et al. 1998; Karapetis et al. 2008). Major mutations of *KRAS* occur in exon 2 (codons 12 and 13). Two phase III clinical trials, OPUS and CRYSTAL, have demonstrated that codon 12 and 13 mutations are predictive for cetuximab activity in combination with FOLFOX or FOLFIRI, respectively (Bokemeyer et al. 2011; Van Cutsem et al. 2011). An additional study also demonstrated that cetuximab combined with first line chemotherapy is effective for patients with *KRAS* G13D tumor mutations (Bokemeyer et al. 2012). A variety of studies have shown that *KRAS* mutations influence the response to the anti-EGFR antibodies cetuximab and panitumumab (Jonker et al. 2007; Karapetis et al. 2008). Several methods have been reported for the detection of *KRAS* mutations in formalin-fixed paraffin embedded (FFPE) tissues (Gonzalez de Castro et al. 2012; Chang et al. 2013; Altimari et al. 2013). The Sanger sequencing method is currently the gold standard (Allegra et al. 2009), however, several *KRAS* mutation kits have become available for colorectal cancer patients.

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