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 (+) 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 plus erlotinib, or a combination of nab-paclitaxel plus erlotinib, a combination of oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLFIRINOX), or a combination of nab-paclitaxel plus erlotinib, a combination of oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLFIRINOX). 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 (TGFβ) receptor II (TGFBR2) and SMAD4 genes are commonly inactivated in several types of cancer, providing evidence that the TGFβ 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 TGFβ-dependent transcriptional regulatory complexes. As a result, TGFβ-mediated growth inhibition is lost. The SMAD4 gene is inactivated in 55% of PC tumors, and numerous studies on the TGFβ 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 TGFβ 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...
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

Su165, Su166, Su167, Su168, Su169, Su170, Su171, Su173, Su174, 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) and were maintained in a 5% CO2-humidified atmosphere at 37°C.

The cDNA fragment encoding the human full-length activin A gene was isolated using PCR and PrimeSTAR HS DNA polymerase (TaKaRa, Otsu, Japan). A full-length cDNA fragment was introduced into a pQCLIN retroviral vector (Clontech; Palo Alto, CA) using a PCR kit (Applied Biosystems, Foster City, CA). The vectors and the stable viral transfectant MIA PaCa-2 and Sui66 cell lines were purchased from Sigma-Aldrich.

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 deviation (SD). The univariate relationship between each independent variable was examined using the χ2 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 820 Y. Togashi et al./Cancer Letters 356 (2015) 819–827 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). Thus, distant metastasis was also analyzed,
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 lines.

### Table 1

Patient characteristics and associations with plasma activin A levels.

<table>
<thead>
<tr>
<th>Patients characteristics</th>
<th>Plasma activin A</th>
<th>( P )-values</th>
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<tbody>
<tr>
<td></td>
<td>&lt;800 pg/mL (n = 23)</td>
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<td>7</td>
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<td>2</td>
</tr>
<tr>
<td>Positive</td>
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<td>4</td>
</tr>
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<tr>
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<tr>
<td>Median OS (days)</td>
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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 \( \chi^2 \) test or the Fisher exact test if there were five or fewer observations in a group.

* \( P < 0.05 \).
In the MIA PaCa-2 cell line (wild-type ACVR1B and SMAD4 gene), however, activin A greatly enhanced cell 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 TGFβ 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 [IC50] of 5-FU; EGFP, 3.36 μM vs. INHBA, 3.62 μM and IC50 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. 51), 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; *\(P < 0.05\).
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 mm³ vs. INHBA, 482.4 ± 182.4 mm³; P = 0.24 on day 22; and EGFP, 447.9 ± 195.0 mm³ vs. INHBA, 549.9 ± 257.7 mm³; 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 TGFβ 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.
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 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; ×200). Ladder, 1 mm; Scale bar, 20 μm.
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
Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.10.037.

References