Overexpression of Fn14 promotes androgen-independent prostate cancer progression through MMP-9 and correlates with poor treatment outcome

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Introduction

Prostate cancer (PCa) is currently the most frequent malignancy in men and the second leading cause of cancer related death in Western populations (1). In Japan, PCa morbidity and mortality have been increasing, presumably due to changes in dietary habits, higher rates of screening and an expanding elderly population. Although initially responsive to androgen ablation therapy, PCa eventually develops into metastatic PCa with tumor progression promoting cell invasiveness, apoptosis and angiogenesis in a TWEAK-dependent or TWEAK-independent manner (5). Recently, high expression of Fn14 has been reported in several human cancers such as esophageal cancer, glioma and pancreatic cancer (6–8). We previously showed that the messenger RNA (mRNA) level of Fn14 was enhanced >3.98-fold under high-fat diet compared with low-fat diet in a mouse LNCaP xenograft model (9). It remains unknown whether TWEAK–Fn14 signaling is causatively associated with the progression of PCa.

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a multifunctional cytokine implicated in the pathogenesis of various human diseases, including cancer (3,4). TWEAK acts by binding to the tumor necrosis factor superfamily member receptor fibroblast growth factor-inducible 14 (Fn14), which is a type I transmembrane protein of 102 amino acids, and has been reported to be associated with tumor progression promoting cell invasiveness, apoptosis and angiogenesis in a TWEAK-dependent or TWEAK-independent manner (5). Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that play a major role in proteolytic degradation of structural components of the extracellular matrix, which plays a role in tumor invasion and metastasis (10,11). In a myoblast and skeletal muscle study, the expression of Fn14 and patients outcomes after radical prostatectomy for localized PCa. We found that high Fn14 promoted the invasion, migration and proliferation of the AIPC cells, and MMP-9 mediated the invasive potential regulated by Fn14 in PC-3 cells. We also identified that high expression of Fn14 in human PCa epithelial cells correlated with poor clinical outcome in patients who underwent radical prostatectomy.

Materials and methods

Cells

The human PCa cell lines LNCaP, PC-3 and DU 145 were purchased from the American Type Culture Collection and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) or Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution.

Reagents and small-interfering RNA constructs

Human recombinant TWEAK and MMP-9 were purchased from Peprotech (London, UK) and R&D Systems (Minneapolis, MN), respectively. MMP-9 inhibitor II was obtained from Calbiochem (San Diego, CA). Small-interfering RNA (siRNA) as a control siRNA, 5'-GAGATTCCA-3' was purchased from Qiagen (Valencia, CA). Sequences of siRNAs used in this study were: Fn14 siRNA1, 5'-CTCAGATGTCCCTGAAATTCCTCA-3'; Fn14 siRNA2, 5'-CGCCCCATCATCATCATTCA-3'; Fn14 siRNA3, 5'-ATCATTCCATCATCATTCA-3' and GLZ Luciferase siRNA as a control siRNA, 5'-ACGTTACCGGGAGATCTTCCA-3'. Transfections of siRNAs were performed using Lipofectamine 2000 (Invitrogen).

Expression vector and transfection

Human full-length complementary DNA clones of Fn14 were purchased from OriGene Technologies (Rockville, MD). The pCMV-AC-Myc vector (Origene) was used as an empty vector. We established Fn14 overexpressing PC-3 (PC-3/Fn14) and its control PC-3 transfected with its empty vector (PC-3/Mock) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Stable clones were selected in 0.5 mg/ml G418 (Invitrogen) and Fn14 expression in transfectedants was verified by western blotting with anti-Fn14 antibody (Cell Signaling Technology, Boston, MA) (Supplementary Figure S1 is available at Carcinogenesis Online).

Western blotting

Cells were lysed by the Complete Lysis-M buffer (Roche, Basel, Switzerland). Equal amounts of proteins were separated by sodium dodecyl sulfate-
polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride filter (ATTO, Tokyo, Japan) and hybridized with corresponding antibodies. Bands were visualized by the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). The following antibodies obtained from Cell Signaling Technology were used: anti-beta-actin, anti-MMP-9 and anti-poly(adenosine diphosphate-ribose) polymerase.

Quantitative reverse transcription–polymerase chain reaction

Total RNA extracted from cells using the TRIzol reagent (Qiagen) was modified to complementary DNA using the SuperScript II reagent (Invitrogen). Each complementary DNA sample was used for the real-time monitoring of quantitative reverse transcription–polymerase chain reaction (RT–PCR analysis). The polymerase chain reaction condition were 90°C for 30 s, 60°C for 30 s and 72°C for 45 s. For the following primers were used: TWEAK, forward 5'-CCCTGCGTCGCTGGAGAAA-3' and reverse 5'-AGACAGGGGGCCCT-CAGTGA-3'; Fn14, forward 5'-CCAGCCTCTCCTACCAAAA-3' and reverse 5'-TGGGCGCTATGTCAAGTCTC-3'; MMP-9, forward 5'-ATTCTGCCAGGACCGCTCTTACT-3' and reverse 5'-CATTTTGATCCGG-CAAATGCTGT-3' and reverse 5'-CTGACTAACCCTGGTGTACACCTCTA-3' and reverse 5'-CGTCAATCTCCTGGTGTACACCTCTG-3'. Relative quantification of gene expression was performed as described previously (15).

Gelatin zymography

MMP-9 expressions in the conditioned medium were determined by a gelatin zymography assay. The conditioned medium was collected and concentrated using an Amicon filter (Millipore, Billerica, MA). Equal amounts of proteins (10 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a gel containing 0.1% gelatin under non-reducing conditions. Next, the gel was washed to remove sodium dodecyl sulfate in 2.5% Triton X-100, by incubating it in a development buffer for 20 h at 37°C. The gelatinolytic activity was visualized after staining with 0.1% Coomassie Brilliant Blue, and the gel was destained in 50% methanol and 10% acetic acid. The bands were quantified using CS Analyzer (2.0) software (ATTO).

Cell proliferation assay

In 96-well plates, 2 x 10³ PCa cells were seeded and the plates were incubated for the time points. The assay was performed using a non-radioactive MTT-based cell proliferation assay kit (Roche) according to the manufacturer's instructions. The absorbance was measured at 570 nm using an ELISA reader (Bio-Rad, Tokyo, Japan). All experiments were conducted in triplicate.

Flow cytometry analysis

Flow cytometric analysis of propidium iodide-stained nuclei was performed, as described previously (16). Briefly, the PC-3 cells were transfected with Fn14 or control siRNA (40 nM) for 24, 48 and 72 h. The cells were trypsinized and fixed in 70% ethanol at –20°C for overnight and then incubated with 1 µg/ml RNase (Qiagen) for 30 min at 37°C before stained with 5 µg/ml of propidium iodide (Sigma, St Louis, MO) at 37°C for 30 min. The stained cells were analyzed for relative DNA content using the FACSCalibur (BD Biosciences, Bedford, MA). Approximately 50 000 cells were examined for each sample. Data were analyzed by CELL Quest software (BD Biosciences). Each assay was performed in triplicate.

Wound healing assay

In a 35 mm dish, 1 x 10⁴ PCa cells were seeded and the plates were incubated for the control time points. The assay was performed using a non-radioactive MTT-based cell proliferation assay kit (Roche) according to the manufacturer’s instructions. The absorbance was measured at 570 nm using an ELISA reader (Bio-Rad, Tokyo, Japan). All experiments were conducted in triplicate.

Matrigel invasion assay

The in vitro invasion assay was carried out in Growth Factor Reduced BD BioCoat Matrigel invasion chambers (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells (1 x 10⁴) were seeded in serum-free medium in the upper compartment, and Dulbecco’s modified Eagle’s medium plus 5% fetal bovine serum was placed in the lower compartment of the chamber as a chemoattractant. After 22 h of incubation, the non-invading cells on the upper side of the chamber were removed, and the membranes were stained with a Diff-Quik cell staining kit (Sysmex, Kobe, Japan). Evaluation of invasive capacity was performed by counting the invading cells under a light microscope. All experiments were conducted in triplicate.

Animal study

The institutional review board of the Akita University School of Medicine approved all animal experiments. Male 6-week-old Balb/c nu/nu mice (Japan SLC, Tokyo, Japan) were used for in vivo experiments. First, subcutaneous xenografts were established for tumor growth assay. An amount of 1 x 10⁶ of either PC-3/Fn14 or PC-3/Mock was inoculated into each of 10 mice subcutaneously in the flank region of the mice with 0.25 ml Matrigel using a 27-gage needle. Tumor volume was measured twice weekly and calculated by the formula: length (cm) x weight (cm) x height (cm) x 0.5236 (9). After the mice were killed at 8 weeks, the Fn14 expression levels in xenografts were assessed by immunohistochemistry. The mRNA levels of MMP-9 in xenografts were analyzed by quantitative RT–PCR. True invasiveness of the cells was determined in vivo using the diaphragm invasion model as described previously (17). Briefly, 1 x 10⁶ of either PC-3/Fn14 or PC-3/Mock cells were inoculated by intraperitoneum injection into each of the 10 mice. Mice were killed at 60 days after injection of cells, and the diaphragms removed from all mice were assessed microscopically under hematoxylin and eosin staining. First, we assessed the presence of diaphragm invasion in all mice. Mice without evident diaphragmatic tumors were excluded from further scoring analysis. The invasiveness was scored semiquantitatively on a 4-point scale measuring the greatest extent of invasion into the diaphragm muscle, with 0 being no invasion to 4 being complete transmigration of the diaphragm.

Tissue collection and immunohistological analysis

Slides containing tissue samples from 112 radical prostatectomy specimens were obtained from the Akita University hospital. The Fn14 rabbit polyclonal antibody (Cell Signaling technology) at a dilution of 1:50 was used as a primary antibody. Immunohistochemical staining was performed as described previously (16). All evaluation and scoring of immunostaining were performed (by S.N. and M.H.) in a blinded manner regarding the patient’s background and clinicopathological information. The Fn14 staining intensity in the cancer epithelium was scored on a semiquantitative scale as follows: 1, low; 2, moderate; and 3, high. The institutional Review Board of the Akita University School of Medicine approved all experiments and human samples were obtained after informed consent.

Statistical analyses

All statistical analyses were performed using Microsoft Excel and SPSS ver 12 software. All the values were presented as mean ± SE. Statistical significance was evaluated by an unpaired Student’s t-test or analysis of variance repeated measurement for comparison between two means in each experiment. Differences were considered statistically significant at P < 0.05.

Results

Expression, sequence-specific inhibition and stimulation of TWEAK and Fn14 in androgen-independent PCa cells

The expression levels of TWEAK and Fn14 in three PCa cell lines (LNCaP, PC-3 and DU 145) were assessed by quantitative RT–PCR and western blotting (Figure 1A and B). The Fn14 mRNA expression level was significantly higher in PC-3 and DU 145 than that of LNCaP cells (P < 0.001, P < 0.001, respectively) (Figure 1A, upper). Similarly, the protein expression of Fn14 was higher in PC-3 and DU 145 compared with that of LNCaP (Figure 1B). Bands of the Fn14 protein on western blotting were in a range from 14 to 17 kDa as described previously by Meighan-Mantha et al. (18). The TWEAK mRNA levels in PC-3 and DU 145 were also significantly higher than that of LNCaP cells (P < 0.001, P < 0.001, respectively) (Figure 1A, under). The mRNA expression levels of Fn14 were similar to that of TWEAK mRNA levels in each PCa cell line. These results suggested that both Fn14 and TWEAK were highly expressed in AIPC cells compared with androgen-sensitive PCa cells. As shown in Figure 1C, the treatment of PC-3 and DU 145 cells with Fn14 siRNAs at 40 nM significantly reduced Fn14 mRNA levels by up to 91 and 78%, respectively. As confirmed by the results of the mRNA study, cells transfected with Fn14 siRNAs displayed sequence specific reduction in the expression level of Fn14 protein (Supplementary Figure S2 is available at Carcinogenesis Online). Control siRNA did not exhibit any effect on the expression of Fn14 mRNA. Furthermore, there were no effects on the expression levels of TWEAK mRNA in PC-3 and DU 145 cells transfected with Fn14 siRNAs (Figure 1C, under). As Fn14 has been shown to be the only tumor necrosis factor family effector modulator by TWEAK (19), we investigated whether the ligand stimulation caused activation of Fn14. By induction of 100 ng/ml human recombinant TWEAK (rTWEAK), both mRNA and protein levels of Fn14 in
PC-3 cells were sequentially increased at up to 24 h (Figure 1D and Supplementary Figure S3, available at Carcinogenesis Online). Collectively, Fn14 knockdown by siRNAs had no effect on TWEAK expression and rTWEAK stimulated Fn14 mRNA expression in AIPC cells. These results suggested that Fn14 acted as a downstream effector in TWEAK signaling in AIPC cells. Based on these results, Fn14 siRNA1, having the highest potential to inhibit Fn14 expression in three Fn14 siRNAs tested in this study, and rTWEAK were used for further functional analysis in vitro.

Modulation of Fn14 influence on invasion and migration in PC-3 and DU 145 cells
To verify the role of Fn14 in cell invasion, we compared the invasive capacity in PC-3 and DU 145 cells treated with either Fn14 siRNA or...
control siRNA (40 nM) by the Matrigel invasion assay. The siRNA-mediated knockdown of Fn14 reduced the invasive capacity in PC-3 and DU 145 cells by 81 and 72% (P < 0.05, P < 0.01, respectively) (Figure 3A). There was no significant difference in the invasiveness in PC-3 and DU 145 cells treated with control siRNA and untreated control cells. Next, to assess the effect of indirect activation of Fn14 on invasive capacity, the Matrigel invasion assay was performed using PC-3 and DU 145 cells stimulated by rTWEAK (0, 50 and 100 ng/ml). The rTWEAK significantly increased the invasive activity of PC-3 and DU 145 cells in a dose-dependent manner (Figure 2B).

Using a wound-healing assay, we compared cell mobility after siRNA transfection. The area of the wound was of no significant difference in PC-3 and DU 145 cells treated with control siRNA and untreated control cells, whereas the area of wound in PC-3 and DU 145 cells treated with Fn14 siRNA significantly decreased at 24 h (P < 0.05, Figure 2C). These findings confirmed that Fn14 modulated the invasive capacity and migration in the two AIPC cell lines.

**Cell proliferation and anti-apoptotic potential of Fn14 in PC-3 cells**

Fn14 has been shown to have antitumorigenic and protumorigenic activity under different conditions (5,20,21). As shown in Figure 3A, Fn14 siRNA-mediated knockdown significantly inhibited the proliferation of PC-3 at 48 h after administration of siRNA compared with PC-3 treated with control siRNA (P < 0.01). In contrast, control siRNA had no effect on cell proliferations of PC-3 compared with untreated control cells. To examine whether the knockdown of Fn14-induced apoptosis in PC-3 cells, we assessed the percentage of apoptotic cells in PC-3 treated with siRNAs by flow cytometry analysis. The fraction of cells undergoing apoptosis (sub-G0–G1 fraction) was significantly higher after treatment of PC-3 cells with 40 nM Fn14 siRNA for 72 h, compared with 40 nM control siRNA (30.1 ± 1.0 versus 9.4 ± 0.3%, P < 0.01, Figure 3B). In addition, the PC-3 cells treated with Fn14 siRNA had a significantly higher amount of cleaved poly(adenosine diphosphate-ribose) polymerase products compared with PC-3 cells treated with control siRNA (P < 0.01, Figure 3C). The growth rate of PC-3/Fn14 was significantly higher than that of PC-3/Mock and untreated control PC-3 cells at 48 h after inoculation of 2 × 10^3 cells (P < 0.01, Figure 3D). Collectively, Fn14 modulated cell proliferation partly through its antiapoptotic potential on PC-3 cells.

**TWEAK–Fn14 enhanced the expression of MMP-9 and the MMP-9 mediated Fn14-regulated invasiveness in PC-3 cells**

Next, we assessed whether TWEAK–Fn14 had an influence on the expression and activity of MMP-2 and MMP-9 in AIPC cells. Using quantitative RT–PCR, we found that MMP-9 mRNA expression was significantly downregulated in PC-3 cells treated with Fn14 siRNA compared with the PC-3 cells treated with control siRNA (P < 0.01), whereas cells treated with control siRNA did not inhibit the expression of MMP-9 compared with untreated PC-3 cells (Figure 4A). Fn14 knockdown had no effects on MMP-2 expression (data not shown). The rTWEAK also stimulated the expression of MMP-9 mRNA in a dose-dependent manner (Figure 4B). Using gelatin zymography, the expression of pro-MMP-9 in a cultured medium of PC-3 treated with Fn14 siRNA was significantly lower than that of control siRNA and untreated PC-3 (Figure 4C, upper). The significant expression of pro-MMP-9 was found in the medium from PC-3 stimulated by rTWEAK in a dose-dependent manner (Figure 4C, under). To determine the role of MMP-9 in the Fn14-enhanced invasive capacity of PCa cells, we assessed the effect of MMP inhibition under Fn14-induced cell invasiveness in PC-3 cells by an MMP-2/9 Inhibitor II. As shown in Figure 4D, cell invasion in PC-3/Fn14 was upregulated by 4-fold compared with that in PC-3/Mock, and the activated invasiveness in PC-3/Fn14 was significantly inhibited by 1 and 5 nM of MMP inhibitor. Furthermore, supplementation with recombinant human MMP-9 overcame the inhibitory effect of Fn14 siRNA on cell invasiveness in a concentration-dependent manner in PC-3 cells (Figure 4E). These results suggested that Fn14 modulated invasive capacity in PC-3 cells in part via MMP-9.

**Acceleration of tumor progression and invasiveness in vivo by Fn14 overexpressing PC-3 cells**

Because we found that Fn14 modulated cell invasive capacity and proliferation in vitro, we next examined whether the findings in vitro...
were similar to be observed in vivo. We used subcutaneous xenografts and invasion models of the diaphragm as described previously (17,22). For the subcutaneous xenografts, $1 \times 10^6$ cells were subcutaneously inoculated and the average volume of tumors measured. At day 52, the rate of tumor growth of the PC-3/Fn14 xenografts was significantly higher compared with that of the PC-3/Mock xenografts ($P < 0.05$, Figure 5A). Higher expression of Fn14 protein in cancer cells of PC-3/Fn14 xenografts compared with PC-3/Mock xenografts was confirmed by immunostaining using anti-human Fn14 antibody (Figure 5B). Furthermore, the mRNA expressions of MMP-9 in PC-3/Fn14 xenografts were significantly higher than those in PC-3/Mock xenografts ($P < 0.05$, Figure 5C).

Mamoune et al. (17) established the diaphragm invasion model of PCa and reported that the semi-quantitative scores of diaphragm invasion correlated with the qualitative assessment of invasiveness of PCa tumors. We excluded three mice, with unsuccessful injection of cells into the abdominal cavity, from further investigation. Mice inoculated with PC-3/Fn14 exhibited a significantly higher incidence of...
invasion into the diaphragm compared with those inoculated with PC-3/Mock xenografts (66.7 versus 37.5%, \( P < 0.01 \), Figure 5D and E). The in vivo analysis confirmed the in vitro results that Fn14 strongly enhanced tumor growth and invasion in AIPC cells.

Association of Fn14 expression with recurrence-free survival in human PCa patients treated by radical prostatectomy

Fn14 staining in human prostate tissues was evaluated by immunohistochemistry using specimens obtained by surgery (Figure 6). Fn14 expression, when present, was most predominantly in the membrane and weaker in the cytoplasm of the cancer epithelial cells. The staining intensity of Fn14 in PCa specimens was divided into three groups according to the intensity and proportion of positive cells. The staining intensity was low in 39 (34.8%) patients, moderate in 61 (54.5%) patients and high in 12 (10.7%) patients (Figure 6A). We assessed the association between clinical outcome after surgery in patients with localized PCa and Fn14 expression level. Kaplan–Meier analysis showed that patients with high Fn14 staining intensity in cancer epithelial cells had significantly shorter prostate-specific antigen recurrence-free survival than patients with low or moderate Fn14 staining intensity (\( P = 0.024 \), Figure 6B).

Discussion

Fn14 has been known to be overexpressed in many types of human cancer cells (6,7,23). Culp et al. (20) investigated Fn14 expressions in 22 solid tumors. Fn14 was overexpressed in a wide range of cancers such as pancreatic, non-small-cell lung and colorectal cancer, whereas there was no strong expression of Fn14 in 61 PCas including three patients with weak expressions (20). In our study, Fn14 was detected in the membranes and cytoplasm with moderate (54.5%) and high (10.7%) levels in PCa specimens. Although these differences might result from a variation of methodology and the affinity of the primary antibody, a further extensive study may be required to clarify the expression and role of Fn14 in human PCa specimens.

One of the significant biological functions of Fn14 that affects cancer aggressiveness is the modulation of invasive capacity. Studies using glioma and esophageal adenocarcinoma cells revealed that the invasive capacity of tumor cells was positively related to upregulated Fn14 levels (5,24). This study revealed that the inhibition of endogenous Fn14 levels using siRNA resulted in a decrease in invasive activity and migration, and the overexpression of Fn14 increased invaded cells in AIPC. We also found that ligand activation of Fn14 by rTWEAK and forced expression by stable transfection of Fn14, upregulated invasive capacity in AIPC. Furthermore, rTWEAK stimulated the expression of Fn14 in AIPC cells in both a dose- and time-dependent manner. These results strongly suggested that activation of TWEAK–Fn14 signaling is associated with progression, especially invasive capacity in AIPC.

Fn14 has been reported to control its downstream signals, which is well known to be associated with cancer progression. Our results showed that MMP-9 was one of the candidate downstream signals to correlate with TWEAK-Fn14 signaling. Excessive production of MMP-2 and MMP-9, two major MMPs, has shown correlations with cancer aggressiveness in PCa (25,26). In mammary gland tumorigenesis, TWEAK has been reported to increase proliferation and invasion.
In this study, we showed that Fn14 enhanced tumor growth sensitive Fn14-expressing tumor cells, suggesting that TWEAK the survival of mice intraperitoneally inoculated with TWEAK−significantly reduced the frequency of tumor rejection and shortened ing has protumorigenic potential in cancer. On the other hand, admin-
glioblastoma cell survival and suggested that TWEAK–Fn14 signal-
et al.
or antitumorigenic effects depending on the particular microenviron-
NF-
and to promote MMP-9 expression (5). Li et al. (13) described that the levels of MMP-9 in myofibers were higher in the TWEAK-transgenic mouse, and TWEAK stimulated MMP-9-promotor activity through nuclear factor-kappaB (NF-κB) and p38 mitogen-activated protein kinase. In PCa, the increased activity of NF-κB and its promotion of apoptotic inhibition is a critical factor in the progression of AIPC (27,28). Further dissection of the functional contribution of TWEAK–Fn14 signaling in tumor progression through candidate coregulators of TWEAK–Fn14 signaling, such as MMPs and/or NF-κB, may provide a new insight into advanced PCa. Recently, the sensitivity of anoikis, which is a unique phenomenon reflecting apotic cell death consequental to insufficient cell–matrix interaction and emerging as a hallmark of metastatic cancer cells, was reported to be regulated by NF-κB and MMP-9 in cancer cells (29,30). It would be exciting to evaluate the relationship between TWEAK–Fn14 and resistance of anoikis in PCa.

Signaling by TWEAK–Fn14 seems to exert either protumorigenic or antitumorigenic effects depending on the particular microenvironment. Tran et al. (8) showed that Fn14 protein functions, partly, by upregulating BCL-XL and BCL-W expression to foster malignant glioblastoma cell survival and suggested that TWEAK–Fn14 signaling has protumorigenic potential in cancer. On the other hand, administration of neutralizing anti-TWEAK monoclonal antibody significantly reduced the frequency of tumor rejection and shortened the survival of mice intraperitoneally inoculated with TWEAK-sensitive Fn14-expressing tumor cells, suggesting that TWEAK mediates the antitumor effect of macrophages through Fn14 in vivo. In this study, we showed that Fn14 enhanced tumor growth in vitro and in vivo, and suppression of Fn14-induced apoptosis. Although the effect of activated TWEAK–Fn14 signaling on tumorigenesis depends on the condition of the microenvironment and type of cancers, Fn14 may enhance tumor growth and have a potential oncogenic activity in AIPC. We previously demonstrated that Fn14 had a potential for involvement in enhanced tumor progression by high-fat diet (9). Several epidemiological studies have suggested that a high-fat diet may be a risk factor of PCa (31,32). Although the precise molecular mechanisms remain unknown, cytokine production may be one of the underlying molecular mechanisms in tumor progression caused by specific diet and diet-induced obesity (33). Chacon et al. reported that TWEAK-derived inflammatory cytokines were increased in severe obesity. It is intriguing to consider testing the hypothesis that diet and obesity promote cancer progression through TWEAK–Fn14 signaling in PCa.

There are several limitations found in our study. Firstly, we did not assess the role of androgen receptor in Fn14-modulated tumor progression because PC-3 and DU 145 cells have no androgen receptor. Even in end-stage PCa, androgen receptor has been known to be overexpressed (34). Therefore, an Fn14 function by an androgen-mediated pathway and the correlation between Fn14 and the androgen receptor in human PCa remains to be elucidated. Secondly, although we investigated the additive effect of Fn14 using PC-3 with overexpressed Fn14, it would be better to use a PCa cell line with less Fn14 expression such as LNCaP cells for an endogenous overexpression model. However, exogenous Fn14 was quite toxic to LNCaP cells and resulted in their death in our experiments. Lastly, although we showed that Fn14 played a significant role in subcutaneous xenograft and direct invasion using the diaphragm invasion assay, further studies involving animal models with an orthotopic intraprostatic implantation and a tail injection of tumor cells are needed to elucidate the effect of Fn14 on distant metastasis.

In summary, the results of the present study suggested that TWEAK–Fn14 signaling has significant oncogenic activities in AIPC, including invasion, migration and proliferation. We suggest that the enhanced tumor progression is mediated by TWEAK–Fn14 at least in part by the enhanced expression of MMP-9.

Supplementary material

Supplementary Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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Fig. 6. Expression of Fn14 in human prostate cancer. (A) Fn14 immunostaining in human PCa. The intensity of Fn14 expression was divided into three groups: low (left), moderate (middle) and high (right). (B) Prostate-specific antigen (PSA) recurrence-free survival was stratified in Fn14 expression. The Kaplan–Meier plot shows the relationship between Fn14 staining intensity in cancer epithelial cells and PSA recurrence rates in localized prostate cancer treated with radical prostatectomy. PSA recurrence was defined as >0.4 ng/ml.

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