

Therapeutic potential of the TWEAK/Fn14 pathway in intractable gastrointestinal cancer

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Abstract. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily. It has been suggested that it plays a pivotal role in various physiological and pathological conditions due to its proinflammatory properties. Fibroblast growth-inducible 14 (Fn14) has been identified as a TWEAK receptor. A number of studies have suggested that TWEAK-Fn14 interaction results in the promotion of apoptosis, cell growth as well as angiogenesis. Although recent studies have indicated that TWEAK and Fn14 are expressed in a number of tumor lines and tissues, the therapeutic potential of this pathway has yet to be elucidated. This study investigated the potential of TWEAK and Fn14 in esophageal and pancreatic cancer as novel molecular targets for anti-cancer therapy. TWEAK and Fn14 protein expression was evaluated in 43 patients with esophageal cancer and 51 patients with pancreatic cancer by immunohistochemistry. As a result, either TWEAK or Fn14 expression was observed in 58.1% of the cases with esophageal cancer and 74.5% of the cases with pancreatic cancer. Furthermore, TWEAK/Fn14 gene expression was identified in the majority of the human esophageal and pancreatic cancer cell lines. Therapeutic efficacies of blocking TWEAK and Fn14 were evaluated by tumor growth inhibition assay in TWEAK- and Fn14-expressing human esophageal and pancreatic cancer cell lines. Coculture with anti-TWEAK or -Fn14 mAb was found to induce a 22-65% cell growth inhibition of these cells. Finally, the significant therapeutic effect of targeting this pathway under *in vivo* physiological conditions was confirmed using a murine

gastrointestinal cancer model. In conclusion, the TWEAK/Fn14 pathway may be functional and critical in intractable gastrointestinal cancers. Therefore, TWEAK and/or Fn14 may be novel molecular targets for anti-cancer therapy.

Introduction

Recent advances in cancer therapy have significantly improved patient prognosis (1). However, a number of gastrointestinal malignancies, including pancreatic and esophageal cancer, remain difficult to treat and are often fatal (2,3). The development of a novel therapeutic approach against these intractable cancers is therefore urgently required. Molecular-targeted therapy may provide a breakthrough in cancer treatment. Although such novel approaches have improved the clinical outcome in several types of cancer, a definitive target for pancreatic and esophageal cancer has yet to be identified.

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK, also known as CD255, TNFSF12 and APO3L) is a member of the TNF superfamily that was originally identified in 1997. It has been suggested that it plays a pivotal role in various inflammatory conditions due to its proinflammatory properties (4,5). Fibroblast growth factor-inducible 14 (Fn14, also known as CD266, TWEAKR and TNFRSF12A) was identified in 2001 as a TWEAK receptor that is linked to several intracellular signaling pathways (5-7). Previous studies have shown that TWEAK-Fn14 interaction promotes cell proliferation, migration, differentiation, apoptosis and angiogenesis as well as inflammation in various cell types (8,9). Furthermore, it has been revealed that this pathway play a key role in a variety of diseases, including rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (10-13). In addition, TWEAK and Fn14 may play roles in certain malignant tumors. Previous studies have demonstrated TWEAK expression in human malignancies in various organs, including the brain, lung, large intestine, liver and breast (4,14,15). Fn14 expression has also been found in tumors in a variety of organs, including the liver, breast, brain, lung, pancreas and large intestine (16-19). Furthermore, a number of functional studies have suggested that TWEAK and/or Fn14 mediate tumor cell apoptosis, proliferation, migration, angiogenesis and survival (20,21). Therefore, it has

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been proposed that TWEAK and Fn14 are potential targets for cancer therapy (5,19,22). However, this strategy requires careful evaluation using *in vitro* and *in vivo* studies since TWEAK/Fn14 appears to exert diverse biological activities in humans (5,23,24). In this study, we evaluated both TWEAK and Fn14 protein expression in actual human esophageal and pancreatic cancer tissues. We further examined the therapeutic potential of targeting the TWEAK/Fn14 pathway against gastrointestinal cancer *in vitro* and *in vivo*.

Materials and methods

Patients. A total of 43 patients with esophageal cancer and 51 with pancreatic cancer who underwent surgery at the Department of Surgery, Nara Medical University, were examined between 1995 and 2004. The esophageal cancers evaluated in this study were pathologically diagnosed as squamous cell carcinoma (SCC). The pancreatic cancers were diagnosed as invasive ductal carcinoma. For immunohistochemistry, each specimen was fixed in 10% phosphate-buffered formalin and embedded in paraffin. A serial section from each specimen was stained with H&E for histological evaluation. The tumors were classified according to the TNM staging system. Written informed consent was obtained from the patients prior to the study according to our institutional guidelines.

Cell lines. Four human esophageal cancer cell lines, TE3 (highly differentiated squamous carcinoma), TE7 (adenocarcinoma), TE8 (moderately differentiated squamous carcinoma) and TE13 (poorly differentiated squamous carcinoma), and four human pancreatic cancer cell lines (CAPAN2, PK-8, PANC-1 and MIAPaCa-2) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. A murine colorectal cancer cell line (colon-26) was obtained from the Riken Cell Bank (Ibaragi, Japan). The cell lines were maintained in Dulbecco's modified Eagle's medium/F12 medium with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂.

Reagents. For the *in vitro* and *in vivo* studies, anti-TWEAK blocking monoclonal antibodies (mAb) (CARL-1; as anti-human and MTW-1; as anti-murine) and anti-Fn14 blocking mAb (ITEM-2; as both anti-human and anti-murine) were prepared as previously described (9,25,26).

Immunohistochemistry. The sections were stained using a Dako EnVision system (Dako Cytomation, Kyoto, Japan), according to the manual provided by the manufacturer. As primary antibodies, rabbit polyclonal anti-TWEAK (FL-249, 1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse monoclonal anti-Fn14 (Clone ITEM-4, 1:100 dilution; eBioscience, San Diego, CA, USA) were employed. After neutralization of endogenous peroxidase, sections were incubated for 90 min with primary antibodies. After three washes in PBS, the sections were incubated for 1 h with polymeric conjugate washed three times with phosphate-buffered saline (PBS). The reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride and the slides were counterstained with hematoxylin.

RT-PCR analysis. Total RNA was isolated from the cell lines using guanidine isothiocyanate methods. RNA was transcribed to cDNA using Omniscript Reverse Transcriptase kit (Qiagen, Hilden, Germany) and oligo dT primers (Amersham Biosciences, Piscataway, NJ, USA). The RNA was heated for 5 min at 65°C and 4°C cooled on ice. Reaction buffer 10X (2 µl), 1 µl of RNase inhibitor (10 U/µl), 2 µl of a mixture of dNTPs, 1 µl of oligodT primers (10 µM), 1 µl of Omniscript Reverse Transcriptase (4 U/µl) and RNase-free water were added for a total volume of 20 µl. The mixture was incubated for 60 min at 37°C. After incubation, the cDNA was stored at -80°C before analysis. The cDNA solution (0.5 µl) was amplified in a total volume of 20 µl that contained 2 µl of 10X PCR buffer, 0.6 µl of MgCl₂ (50 mM), 0.2 µl of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), sense primer (50 µM), antisense primer (50 µM) and 2 µl of a mixture of dNTPs (Takara, Ohtsu, Japan). The amplification involved an initial step of denaturation at 94°C for 3 min, followed by cycles consisting of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and chain elongation at 72°C for 1 min. The sequences of sense and antisense primers, and the product size for TWEAK, Fn14 and β-actin were: TWEAK, sense 5'-CCCTGCGCTGCCTGGAGGAA-3' and antisense 5'-AGACCAGGGCCCCCTCAGTGA-3' (amplicon size 200 bp); Fn14, sense 5'-CCAAGCTCCTCCAACCACAA-3' and antisense 5'-TGGGGCCTAGTGTCAGTCT-3' (amplicon size 242 bp); β-actin, sense 5'-ATCAAGATCCTGACCGAGCG-3' and antisense 5'-TACTTGCGCTCAGGAGGAGC-3'. The amplified products were analyzed by electrophoresis of the PCR product on a 2% agarose gel plus ethidium bromide in TBE buffer.

Cell growth inhibition assay. Human cancer cells were added at a concentration of 1x10³ cells in 100 µl to the individual wells of 96-well flat-bottomed microtiter plates. Then, 25 µg/ml of anti-human TWEAK mAb (CARL-1), anti-Fn14 mAb (ITEM-2) and control IgG were added to each well every other day and were further incubated at 37°C in a humidified atmosphere of 5% CO₂. After 3 or 7 days, the cells were rinsed with PBS, fixed with 5% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) in PBS and then stained with 0.2% crystal violet (Wako, Osaka, Japan) and 100 mM CAPS (Wako). The absorbance of each well was measured at 540 nm with Multiskan MS (LabSystems, Helsinki, Finland). The growth inhibition rate was calculated using the following formula: % of growth inhibition rate = absorbance of mAb-treated cells - spontaneous absorbance/absorbance of control cells - spontaneous absorbance. The samples were examined in triplicate.

Animal study. Female BALB/c mice (8 weeks old) were obtained from Clea Japan (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions in the animal facility at Nara Medical University. The experiments were conducted under a protocol approved by our institutional review board. Cells (1x10⁶ murine colorectal adenocarcinoma colon-26) were subcutaneously inoculated on one side of the ventral surface in the lower flank region of syngeneic BALB/c mice. Treatment was started 7 days later when a small palpable lump was evident, ranging from 4 to 6 mm in diameter. Some mice were intraperitoneally injected with 0.3 mg of each mAb every other day for 3 weeks. The control mice received control

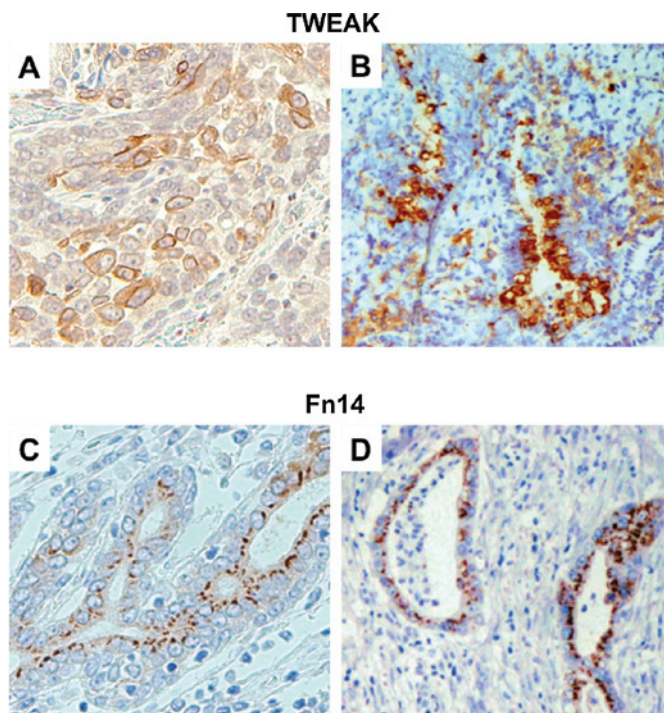


Figure 1. Immunohistochemical staining of human esophageal and pancreatic cancer tissues with FL-249 recognizing (top) TWEAK and ITEM-4 recognizing (bottom) Fn14. (A) Representative case of TWEAK-positive esophageal cancer, (B) TWEAK-positive pancreatic cancer, (C) Fn14-positive esophageal cancer and (D) Fn14-positive pancreatic cancer. Original magnification, x200.

IgG. The tumor size was determined by caliper measurements. The tumor volume was calculated according to the formula: $V = A \times B^2/2$ (mm³), where A is the largest diameter (mm) and B is the smallest diameter (mm).

Statistical analysis. The experimental data of the growth inhibition assay were analyzed by the Student's t-test. Differences of $P < 0.05$ were considered to indicate statistical significance.

Results

TWEAK and Fn14 expression in human esophageal and pancreatic cancer. TWEAK and Fn14 protein expression in actual human esophageal cancer and pancreatic cancer tissues was examined by immunohistochemical staining. In both cancer tissues, TWEAK expression was identified in the cytoplasm and membrane of cancer cells, and Fn14 was observed mainly in the tumor cellular membrane (Fig. 1). In esophageal cancer, 20/43 cases (46.5%) stained positively for TWEAK and 10 cases (23.2%) were positive for Fn14. Overall, either TWEAK or Fn14 expression was observed in 25 cases (58.1%) (Table I). In pancreatic cancer, 18/51 (35.3%) stained positively for TWEAK and 35 (68.6%) were positive for Fn14. Furthermore, 15 (29.4%) cases showed positive staining for both TWEAK and Fn14. Overall, either TWEAK or Fn14 expression was observed in 38 cases (74.5%) (Table II).

TWEAK and Fn14 mRNA expression in human esophageal and pancreatic cancer cell lines. The gene expression of TWEAK and Fn14 was examined in four cell lines of each cancer type.

Table I. Correlation between TWEAK and Fn14 expression in esophageal cancer.

	TWEAK		
	Positive (%)	Negative (%)	Total (%)
Fn14			
Positive	5 (11.6)	5 (11.6)	10 (23.2)
Negative	15 (34.9)	18 (41.9)	33 (76.8)
Total	20 (46.5)	23 (53.5)	43 (100.0)

Table II. Correlation between TWEAK and Fn14 expression in pancreatic cancer.

	TWEAK		
	Positive (%)	Negative (%)	Total (%)
Fn14			
Positive	15 (29.4)	20 (39.2)	35 (68.6)
Negative	3 (5.9)	13 (25.5)	16 (31.4)
Total	18 (35.3)	33 (64.7)	51 (100.0)

As a result, RT-PCR analysis indicated that both TWEAK and Fn14 were detected in all tested esophageal cell lines. In human pancreatic cancer cell lines, TWEAK was expressed in 3/4 cell lines (CAPAN2, PANC-1 and MIA PaCa-2), and Fn14 was observed in all the cell lines (Fig. 2).

Blockade of the TWEAK/Fn14 pathway inhibits gastrointestinal tumor growth. For the clinical application of targeting TWEAK/Fn14 pathway, the therapeutic efficacy of its blockade on gastrointestinal cancer cell growth was evaluated using a number of TWEAK- or Fn14-expressing human cancer cell lines. After co-culture with mAb, TWEAK blockade had an inhibitory effect on both esophageal and pancreatic cancer growth (Fig. 3). The reduction rates of cell growth on the 7th day were: TE3, 22%; TE7, 38%; CAPAN2, 53%; PANC-1, 47%; and MIA PaCa-2, 47%. Furthermore, the Fn14 blockade exerted a similar effect on the cell growth of both types of cancer (reduction rate: TE3, 52%; TE7, 50%; CAPAN2, 55%; PANC-1, 49% and MIA PaCa-2: 65%) (Fig. 4).

TWEAK/Fn14 blockade inhibits gastrointestinal cancer growth in vivo. In order to confirm the therapeutic efficacy of targeting TWEAK/Fn14 in gastrointestinal cancer, a physiological *in vivo* model was employed. To this end, using the murine colorectal adenocarcinoma colon-26 cell line, the efficacy of TWEAK/Fn14 blockade in wild-type BALB/c mice was evaluated. The treatment of anti-TWEAK or -Fn14 blocking mAb significantly inhibited tumor growth in the treated mice (Fig. 5). Thus, the beneficial effect of either TWEAK or Fn14 blockade on gastrointestinal cancer was confirmed even in immunocompetent mice.

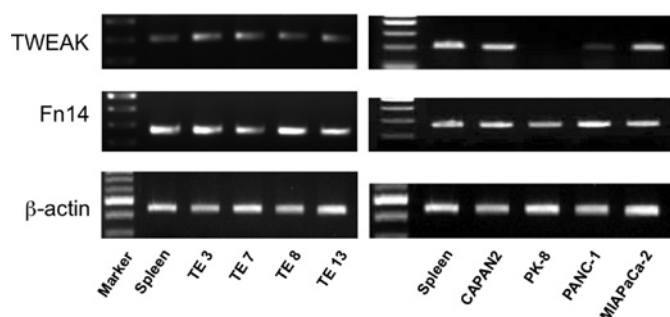


Figure 2. TWEAK and Fn14 mRNA expression in human esophageal and pancreatic cancer cell lines examined by reverse transcriptase-PCR. A 200-bp human TWEAK sequence, a 242-bp human Fn14 sequence and a β -actin sequence were amplified from total RNA, separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

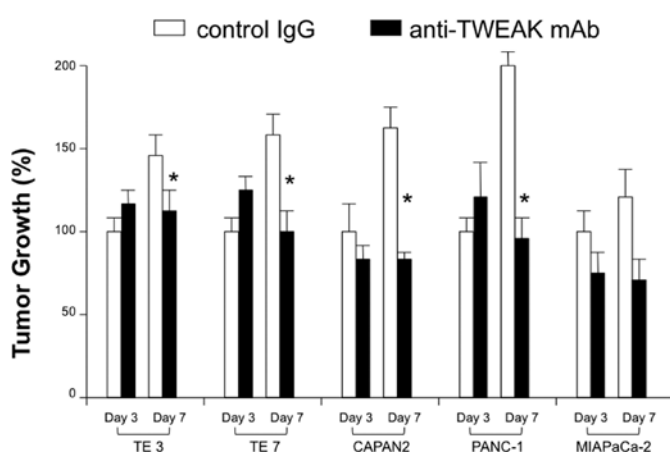


Figure 3. Growth inhibition of esophageal and pancreatic cell lines by anti-TWEAK mAb (25 mg/ml). The cancer cells were co-cultured with either control IgG (white boxes) or anti-TWEAK mAb (black boxes). *Indicates statistical difference in the growth of tumor cells treated with anti-TWEAK mAb compared to the controls. The results are expressed as the mean number of three wells \pm SD obtained from one representative of three individual experiments.

Discussion

TWEAK is a multifunctional cytokine that controls various cellular activities. TWEAK functions by binding the cell-surface receptor, Fn14. Recent cumulative evidence has revealed that the TWEAK/Fn14 pathway regulates a variety of physiological and pathological conditions (5). Although it has been suggested that this pathway is involved in tumor development and progression, the therapeutic potential of targeting TWEAK/Fn14 has yet to be elucidated (14-16,19,22,27). In this study, we evaluated the clinical usefulness of TWEAK/Fn14-targeted therapy in esophageal and pancreatic cancer in representative intractable gastrointestinal tumors.

First, we examined the protein expression of TWEAK/Fn14 in actual human cancer tissues. We confirmed this expression in approximately 60% of esophageal and 75% of pancreatic cancer cases. Although TWEAK protein expression has not been previously evaluated in esophageal and pancreatic cancer, it was recently reported that Fn14 expression is positive in 27% of esophageal cancer and 60% of pancreatic

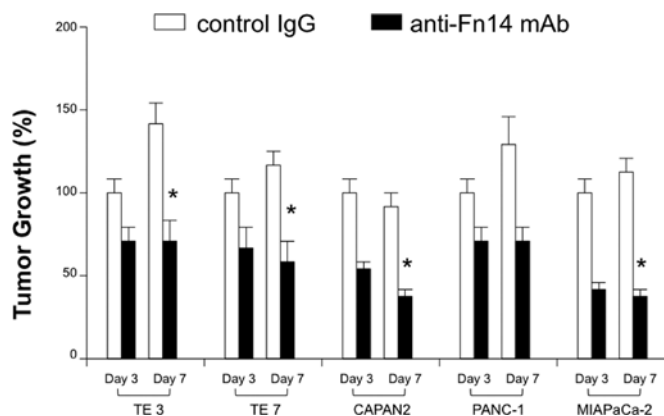


Figure 4. Growth inhibition of esophageal and pancreatic cell lines by anti-Fn14 mAb (25 mg/ml). The cancer cells were co-cultured with either control IgG (open boxes) or anti-Fn14 mAb (closed boxes). *Indicates statistical difference in the growth of tumor cells treated with anti-Fn14 mAb compared to the controls. The results are expressed as the mean number of three wells \pm SD obtained from one representative of three individual experiments.

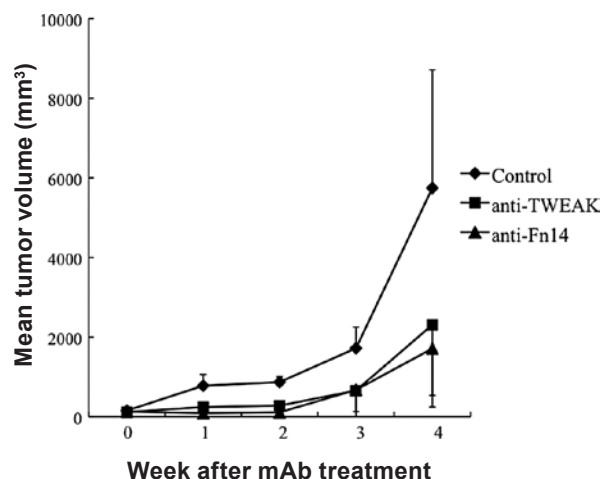


Figure 5. Therapeutic efficacy of targeting TWEAK/Fn14 in gastrointestinal cancer. The effect of TWEAK/Fn14 blockade on gastrointestinal cancer was examined *in vivo*. Colon-26 was implanted in the flank of the syngeneic BALB/c mice. The mice were treated with 0.3 mg of each mAb every other day for 3 weeks. The control mice received control IgG. The mean tumor volume at 1, 2, 3 and 4 weeks after starting treatment was; anti-TWEAK mAb, 243, 279, 656 and 2,311 mm³, respectively; anti-Fn14 mAb, 91, 111, 676 and 1,713 mm³, respectively; control, 781, 870, 1,722 and 5,747 mm³, respectively. $n=6$ for each group. Data points, mean tumor size; bars, SD. Either TWEAK or Fn14 blockade inhibited tumor growth compared to the controls. The statistical differences were; TWEAK blockade, 0.005, <0.0001, 0.02 and 0.07 at 1, 2, 3 and 4 weeks, respectively, compared to the control; Fn14 blockade, 0.0007, <0.0001, 0.06 and 0.02, respectively, compared to the control.

cancer cases (19). In our study, it was positive in 23 and 68%, respectively. These data appear similar, even though there are racial and tumor histological differences. Furthermore, most of the esophageal and pancreatic cancer cell lines examined in this study demonstrated both TWEAK and Fn14 expression. Notably, Fn14 mRNA expression was recently reported as one of the best markers for the early diagnosis of esophageal cancer (28). The TWEAK/Fn14 pathway is therefore a promising novel target for intractable gastrointestinal cancer.

Next, we investigated the therapeutic potential of targeting the TWEAK/Fn14 pathway in gastrointestinal cancer both *in vitro* and *in vivo*. We found that TWEAK and Fn14 blockade had a substantial effect on the inhibition of gastrointestinal cancer. Our *in vitro* experiments clearly demonstrated that TWEAK and Fn14 blockade significantly inhibited cancer cell growth. Thus, data indicate that both molecules are directly involved in the tumor proliferation of esophageal and pancreatic cancer. Furthermore, *in vivo* data evaluated in physiological conditions also demonstrate that TWEAK and/or Fn14 are functional and may be potential targets for a novel anti-cancer therapy. However, due to the diverse functions of TWEAK/Fn14, there may be a number of caveats for clinical application as a molecular-targeted therapy. Tumor cell apoptosis is essential for the induction of therapeutic efficacy in a variety of cancer treatments. TWEAK/Fn14 has been found to be a critical regulator to promote the apoptosis of certain cells under various circumstances (4,5,29). Therefore, its blockade may result in the promotion of resistance to treatment-related apoptosis of tumor cells. Furthermore, recent studies have suggested that TWEAK/Fn14 plays a critical role in tissue repair and regeneration (18,30-32). Therefore, inhibition of this pathway may have detrimental effects or be harmful for cancer patients.

On the other hand, accumulating evidence suggests the therapeutic potential of TWEAK and/or Fn14 blockade as a novel anti-cancer therapy. Soluble TWEAK has previously been shown to promote tumor growth and angiogenesis (21). In addition, Fn14 overexpression has been demonstrated to induce tumor cell migration (20,22). Furthermore, it has been shown that tumor invasion was significantly inhibited by Fn14 blockade using the RNA interference method (16,31). More recently, newly developed antibodies for targeting Fn14 have been demonstrated to inhibit tumor growth through direct inhibition and antibody-dependent cellular cytotoxicity (19). Our *in vitro* and *in vivo* therapeutic data further support a potential of blocking the TWEAK/Fn14 pathway in cancer treatment. A number of potential underlying mechanisms are involved in anti-TWEAK and anti-Fn14 mAb treatment. These include direct inhibition of proliferation, migration, apoptosis, angiogenesis and survival of tumor cells (5,22). Also, antibody-dependent mechanisms may play a critical part *in vivo*, in clinically relevant physiological conditions (19). Further studies are required to clarify the possible mechanisms of the antitumor effect induced by targeting TWEAK/Fn14 in intractable gastrointestinal cancer.

It is crucial to evaluate the combination therapy of targeting TWEAK and Fn14 with conventional antitumor treatments, including chemotherapy and radiotherapy, since chemotherapy and radiotherapy influence the multifunction of TWEAK and Fn14, leading to either a synergistic or detrimental antitumor effect. Particularly in intractable gastrointestinal tumors including esophageal and pancreatic cancer, multimodal treatment appears to be essential for improving patient prognosis. Therefore, further research is warranted to evaluate the combination therapy with current standard chemotherapy, since new molecular-targeted therapies have been reported to enhance the clinical outcome of conventional chemotherapy even in intractable cancer (33). To date, no studies address these issues in regard to the TWEAK/Fn14 pathway.

In conclusion, we demonstrated TWEAK/Fn14 expression in human esophageal and pancreatic cancers representative of intractable gastrointestinal malignancies. Our data indicate that the TWEAK/Fn14 pathway is a novel molecular target for anti-cancer therapy and may provide the rationale for developing this novel strategy against such diseases.

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