

Overexpression of Tropomyosin-Related Kinase B in Metastatic Human Pancreatic Cancer Cells

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ABSTRACT

Purpose: Pancreatic adenocarcinoma is currently the fourth leading cause of cancer death in the United States, and most pancreatic cancers develop locally advanced disease or metastasis at the time of diagnosis. The mechanisms by which it invades and metastasizes are not known.

Experimental Design: To identify the genes involved in pancreatic cancer metastasis, we analyzed the gene expression profiles between highly metastatic Colo357L3.6pl and parental Colo357FG pancreatic cancer cell lines using cDNA microarrays and confirmed differential gene expression by reverse transcription-PCR, Western blotting, and immunologic analysis of 54 samples from pancreatic cancer patients. The correlation with clinical outcome was also examined. The possible signaling pathways involved with tropomyosin-related kinase B (TrkB) were analyzed.

Results: Our findings showed that TrkB was overexpressed in the highly metastatic Colo357L3.6pl cells, which correlated with perineural invasion ($P = 0.026$), positive retroperitoneal margin ($P = 0.0005$), and shorter latency to development of liver metastasis (Cox proportional hazard ratio, 0.3; 95% confidence interval, 0.1–0.8; $P = 0.01$) in patient samples. Extracellular signal-regulated kinases 1 and

2 were activated and Elk-1 and AP-1 DNA binding activity was induced in Colo357L3.6pl cells. Furthermore, interleukin 8 and vascular endothelial growth factor were more strongly expressed in Colo357L3.6pl than Colo357FG cells, and these findings were confirmed in Colo357L3.6pl and Colo357FG orthotopic tumors.

Conclusion: These results suggest that overexpression of TrkB and activation of mitogen-activated protein kinase and AP-1, which may in turn induce the expression of vascular endothelial growth factor and interleukin 8, may mediate the cardinal clinical features of locally aggressive growth and metastasis of pancreatic cancer. Our results also imply that TrkB receptor may be a novel therapeutic target for pancreatic cancer.

INTRODUCTION

Pancreatic adenocarcinoma is currently the fourth leading cause of cancer death in the United States (1). The 5-year survival rate continues to be dismal (1–3%; ref. 1). Current chemotherapy, radiation therapy, and surgical procedures are largely ineffective in the treatment of this disease (2). Surgery helps a very few patients; however, most pancreatic cancers have developed into locally advanced unresectable disease or metastasized at the time of diagnosis and so are inoperable (2, 3). The survival of patients with potentially resectable pancreatic cancer can be increased by using chemoradiation plus pancreaticoduodenectomy (2, 4).

Severe back pain is very common in pancreatic ductal adenocarcinoma and correlates strongly with perineural invasion (5, 6). Back pain is an ominous clinical sign in patients with ductal pancreatic cancer, as the pain indicates irresectability and poor long-term prognosis, even if it develops after curative resection (6–8). In 50% to 90% of all cases, pancreatic cancer cells infiltrate neural structures (5, 6). This infiltration occurs in the retroperitoneal region and may account for local recurrence after tumor resection, which usually involves a negative margin only a few millimeters wide (5). Expression of nerve growth factor correlates with perineural invasion and pain in human pancreatic cancer (9, 10). However, the molecular mechanisms for the frequent perineural invasion in pancreatic cancer and the role of nerve growth factor overexpression in the pathogenesis of the back pain associated with pancreatic cancer are still not clear and need to be elucidated.

The neurotrophin (NT) family consists of nerve growth factor, brain-derived neurotrophic factor, NT-3, NT-4 (also called NT-5), NT-6, and NT-7 (11, 12). These factors act through a group of receptors that includes NT receptor p75 (p75NTR), TrkA, TrkB, and TrkC, which have different affinities for the various ligands (13). Ligand engagement of Trk has been shown to result in phosphorylation of cytoplasmic tyrosine residues in these receptors, which promotes signaling by creating docking sites for adapter proteins containing phosphotyrosine-binding or src-homology-2 (SH-2) motifs (14, 15). These adapter molecules couple Trk to intracellular signaling cascades, including

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the Ras/extracellular signal-regulated kinase (ERK), phospholipase C- γ , phosphatidylinositol-3 kinase (PI-3 kinase), and Fos/Jun and NF- κ B signaling and Jun kinase activation (16). A number of reports showed that persistent Trk activity was necessary to maintain AP-1 activity (17–19).

NT and their receptors have also been shown to play a plethora of roles in malignant cells, including stimulating chemotaxis, tumor invasiveness, clonal growth, and altering cell morphology, in a variety of cancers such as medulloblastomas, neuroblastomas, and lung, thyroid, breast, lymphoma, pancreatic cancer, and soft tissue tumors (20–24). Interestingly, the expression and prognostic significance of NTs and their receptors in cancer development varies among different tissue types. For example, neuroblastomas expressing high levels of TrkA or TrkC are generally less advanced and have a more favorable prognosis (25, 26). However, tumors that express high levels of TrkB and its ligand brain-derived neurotrophic factor have a much poorer prognosis. These findings suggest a function for specific NT receptors beyond the central nervous system.

Bruns et al. generated the highly metastatic Colo357L3.6pl pancreatic cancer cell line, a variant of the parental Colo357FG cell line expressing a mutated *K-ras*, using an orthotopic nude mouse model for the study of molecular mechanisms involved in pancreatic cancer invasion and liver metastasis (27–30). The increased liver metastatic potential of Colo357L3.6pl cells was associated with increased expression of basic fibroblast growth factor, vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), and collagenase type IV (27, 29, 31). To provide a better understanding of the molecular basis of pancreatic cancer invasion and metastasis, we analyzed the differential gene expression profiles between Colo357L3.6pl and Colo357FG cell lines by microarray analysis. Our results show that TrkB overexpression significantly correlated with perineural invasion, positive retroperitoneal margin, and shorter time to liver recurrence and suggest that TrkB receptor overexpression may play an important role in invasiveness and metastasis of pancreatic cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human pancreatic adenocarcinoma cell lines Colo357FG and Colo357L3.3pl, Colo357L3.6pl were previously described (27), and AsPc-1 and CFPanc-1 from the American Type Culture Collection (Manassas, VA). The cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin (Life Technologies), and 10 mg/mL streptomycin (Life Technologies) at 37°C in a humidified 5% CO₂/95% air atmosphere, and harvested at about 75% confluence.

Mice

Female athymic BALB/c nude mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). One million viable Colo357FG and Colo357L3.6pl cells suspended in 50 μ L of PBS were carefully injected into the pancreatic parenchyma of each mouse, so that no tumor cells leaked from the injection site according to previous report (27). Briefly, the mice were housed in cabinets with laminar flow under specific pathogen-free

conditions and were maintained according to institutional regulations in facilities approved by the Association for the Assessment and Accreditation of Laboratory Animal Care, in accordance with the current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the NIH. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas M.D. Anderson Cancer Center. The tumor formation in pancreas and liver metastases in liver was determined 8 weeks after tumor injection.

cDNA Microarray Analysis

We used the Atlas human cancer cDNA expression array from Clontech (Palo Alto, CA). This array contains 588 human cDNAs, including nine housekeeping cDNA and negative controls. RNA was isolated from Colo357L3.6pl and Colo357FG cells by using the Trizol reagent (Life Technologies) as previously described (32). The total RNA was further purified and the cDNA probes synthesized as described in the Atlas user manual except that the cDNA probes were synthesized by using 50 μ g of total RNA from Colo357L3.6pl and Colo357FG cells. The probes were then hybridized to the Atlas arrays as recommended in the user manual. After autoradiography and phosphoimaging, the two membranes were stripped by boiling 0.1 \times SSC buffer for 5 minutes and rehybridized with the swapped cDNA probes. The coordinate maps and descriptions of their corresponding genes were obtained from the Product Analysis Certificate supplied by Clontech. cDNA microarray analyses are repeated twice.

Reverse Transcription-PCR

RNA was obtained from both human pancreatic ductal adenocarcinoma cell lines as previously described (32). The RNA was reverse transcribed into cDNA by reverse transcriptase. The cDNA was amplified by PCR (30 cycles of 1 minute at 94°C, 1 minute at 50°C, and 2 minutes at 72°C) with primers corresponding to TrkB cDNA. Sense 5'-AGTCGCA-GATGCTGCATATTA G-3' and antisense 5'-TAGAATGTC-CAGGTAGACC-3'. In the same reverse transcription-PCR reaction, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified with a pair of GAPDH primers as an internal control.

Western Blot Analysis

Cells were lysed at 4°C in 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethyl sulfonyl fluoride (Sigma Chemical Co., St. Louis, MO), and 25 mmol/L Tris (pH 7.5), and the lysates were cleared by centrifugation. Each lysate (50 μ g of protein) was mixed with an equal volume of 2 \times sample buffer and boiled for 5 minutes. For the VEGF Western blot, 50 μ g of protein from the media conditioned by Colo357L3.6pl cells and Colo357FG cells were used. The samples were separated by 8% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Osmonics, Westborough, MA). The membrane was blocked with 5% nonfat milk and 3% bovine serum albumin in PBS containing 0.2% Tween 20 and incubated with polyclonal rabbit antibodies against TrkA, TrkB, TrkC, VEGF, Erk-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or with monoclonal mouse antibodies against phosphorylated Erk or β -actin (Santa Cruz Biotechnology). Membranes were

washed in PBS containing 0.2% Tween 20 and probed with horseradish peroxidase-coupled secondary goat anti-rabbit or anti-mouse antibodies (Amersham, Arlington Heights, IL). The proteins were visualized with Lumi-Light Western blotting substrate (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Tissue Samples

Tissue samples from 54 patients and normal controls were obtained from the Pancreatic Tumor Tissue Bank of M.D. Anderson after institutional review board approval was obtained. All of the patients had undergone pancreaticoduodenectomy for ductal adenocarcinoma of the pancreas without prior chemotherapy or radiotherapy between 1990 and 2000. All these pancreatic cancer patients had follow-up CT scans every 3 to 4 months for the first two postoperative years and every 6 months for years 3 to 5. The tissues were fixed in 10% buffered formalin and paraffin embedded. A representative H&E stained slide was obtained from each sample and the presence of pancreatic adenocarcinoma confirmed by M.D. Anderson pathologists. Mouse tissue samples from orthotopically growing intrapancreatic Colo357FG and Colo357L3.6pl tumors and liver metastasis of Colo357L3.6pl tumors were processed by the same procedure.

Immunostaining

Paraffin-embedded tissues were used for identification of TrkB, VEGF, and IL-8. Immunohistochemical staining procedures were done by using polyclonal rabbit antibodies against TrkB and VEGF and a monoclonal antibody against IL-8 (Santa Cruz Biotechnology) as previously described (33). Positive immunoreactivity for TrkB, VEGF, and IL-8 was defined as brown-colored precipitate in the cell membrane and cytoplasm. Control samples exposed to secondary antibody alone showed no specific staining. TrkB expression in the human pancreatic cancer samples was assessed by a pathologist not aware of the patients' clinical status. TrkB, VEGF, and IL-8 expressions were assessed in the mouse pancreatic tumor tissues and liver metastases. Quantification of immunostainings was done by averaging two sets of independently obtained results from five microscopic fields (34). The area of tumor stained was determined with a four-tiered scale 0 to 3: "0", no detectable staining; "1", >0 but <20% of tumor cells stained; "2", 20% to 50% of tumor cells stained; "3", >50% of tumor cells stained. The intensity of staining was evaluated with a four-tiered scale 0 to 3 applying the following scoring criteria: "0", no detectable staining; "1", weak staining of tumor cells; "2", moderate staining of tumor cells; "3", strong staining of tumor cells. Finally, the results of the intensity of staining and area were calculated for semiquantitating the immunostainings.

Clinicopathologic Correlation

The results from immunohistochemical analysis for TrkB were correlated with histopathologic and clinical patient characteristics from a continuously updated database of surgically treated pancreatic cancer patients at M.D. Anderson after institutional review board approval was obtained. The database contains demographic data, surgical details, pathology reports, and clinical outcome.

Statistical Analysis

All statistical analyses were done with NCSS software (NCSS, Kaysville, UT). The significance of the data was determined by using the χ^2 test and Cox proportional hazard regression. $P < 0.05$ was considered significant. To estimate the degree of association between variables, odds ratio, and hazard ratio, and corresponding 95% confidence intervals were computed as appropriate.

Electrophoretic Mobility Shift Assay

An electrophoretic mobility shift assay was done with nuclear extracts as previously described (35). The ^{32}P -labeled probes used were double-stranded oligonucleotides for the Elk-1 (5'-GGATGTCCATATTAGGACATCT-3'), AP-1 (5'-CGTTGATGACTCAGCCG GAA-3'), and Oct-1 (5'-TGTCGAATGCAAATCACTAGAA-3') consensus sequence (Sigma, The Woodlands, TX). Equal loading of nuclear extracts was monitored by assessment of Oct-1 binding.

Kinase Assay

Phosphorylation of TrkB was measured in an *in vitro* kinase assay as previously described (36). Briefly, TrkB was immunoprecipitated and the kinase reaction was carried out in 20 μL ATP buffer (50 mmol/L HEPES [pH 7.5], 5 mmol/L MgCl_2 , and 2 mmol/L ATP; ref. 37). After incubation at 37°C for 20 minutes, the reaction products were resolved by SDS-PAGE, dried, and exposed to X-ray films.

Transfection and Luciferase Assay

One microgram of wild type-AP-1 or mutant AP-1 reporter plasmid containing the *firefly* luciferase reporter gene and a pRL-TK plasmid containing the *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase promoter as an internal control were cotransfected into tumor cells in triplicate by using the lipotransfection method (FuGENE 6; Roche) according to the manufacturer's recommendation. The activities of both firefly and *Renilla* luciferase were determined 48 hours after transfection with the dual luciferase reporter assay system (Promega, Madison, WI). The firefly luciferase activities were normalized to the control *Renilla* luciferase activity.

RNase Protection Assay

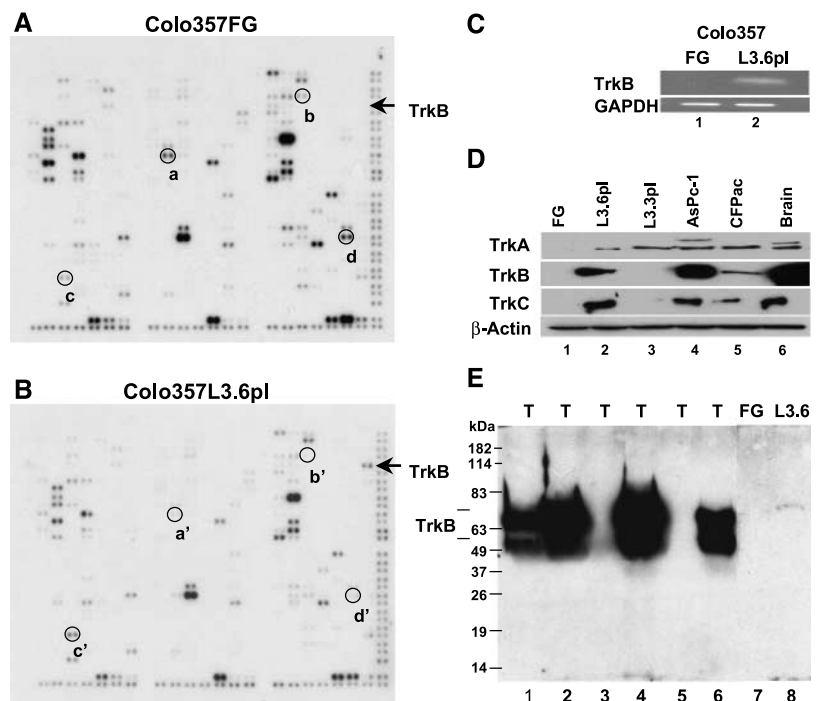
RNA was isolated from Colo357FG and Colo357L3.6pl cells by using TRIZOL Reagent (Life Technologies). A RNase protection assay was done with the Riboquant multiprobe protection assay system (PharMingen, San Diego, CA) according to the manufacturer's recommendation. Quantification was carried out by using phospho-image analysis.

RESULTS

Overexpression of TrkB in Colo357L3.6pl Cells

To search for candidate genes involved in pancreatic tumor invasion and metastasis, we did cDNA microarray analysis to identify genes differentially expressed in the highly metastatic cell line Colo357L3.6pl and its parental cell line Colo357FG. As shown in Fig. 1A and B, the expression of the genes encoding IAP repeat-containing 2 (a, a'), ubiquitin-conjugating enzyme E2A (RAD6 homologue; b, b'), and platelet-derived growth factor- β polypeptide (v-sis) oncogene homologue (d, d') was

Fig. 1 Overexpression of TrkB mRNA and protein in Colo357L3.6pl cells. **A** and **B**, cDNA microarray analysis of Colo357L3.6pl and Colo357FG cells with Atlas human cancer cDNA array 1. Arrows, expression pattern of TrkB (Genbank accession no. M62219). Circle, expression pattern of IAP repeat-containing 2 (a, a'; Genbank accession no. U45879), ubiquitin-conjugating enzyme E2A (RAD6 homologue; b, b'; Genbank accession no. M74524), platelet-derived growth factor- β polypeptide (*v-sis*) oncogene homologue (d, d'; Genbank accession no. X02811), and integrin $\alpha 3$ (c, c'; Genbank accession no. M59911). The microarray analysis was done as described in Material and Methods. Representative autoradiogram. **C**, reverse transcription-PCR analysis confirming the overexpression of TrkB in Colo357L3.6pl cells. GAPDH served as an internal control. **D**, Western blot analysis of TrkA, TrkB, and TrkC in Colo357FG and Colo357L3.6pl cell lines with Trk receptor expressing AsPc-1 and CFPanc-1 cell lines, and mouse brain protein extract as positive controls as indicated. The levels of β -actin were used as a loading control. **E**, Western blot analysis of TrkB in the protein extracts from six human pancreatic cancer specimens, and Colo357FG and Colo357L3.6pl cells as positive controls.



down-regulated in Colo357L3.6pl cells, whereas integrin $\alpha 3$ (c, c') and TrkB were overexpressed in Colo357L3.6pl cells. Quantification by phosphoimaging revealed that expression of TrkB was about 10-fold higher and expression of integrin $\alpha 3$ was about 2-fold higher in Colo357L3.6pl cells than that in Colo357FG cells, and the overall hybridization signals were normalized with GAPDH. Hybridization signals were slightly stronger in membrane hybridized with the Colo357FG cDNA probes and this may be due to differences in the efficiency of RT reactions for different RNA preparations. To verify the cDNA array data, we switched Atlas human cancer cDNA array membranes during rehybridization. The same gene expression patterns as shown in Fig. 1A and B were obtained. To verify these findings, we did further analyses. The overexpression of TrkB in Colo357L3.6pl cells was confirmed by a reverse transcription-PCR assay (Fig. 1C). Western blot analysis was done on Colo357FG, Colo357L3.6pl, and Colo357L3.3pl with Trk receptor expressing AsPc-1 and CFPanc-1 cell lines, and mouse brain protein extract as positive controls (7). Immunoblotting was also done on the protein extracts from human pancreatic adenocarcinoma tissues. As shown in Fig. 1D and E, TrkB protein was overexpressed in the Colo357L3.6pl, four of six pancreatic adenocarcinoma samples and positive control cell lines AsPc-1 and CFPanc-1, but not in Colo357FG cells. Interestingly, TrkB protein was also not overexpressed in the cell line Colo357L3.3pl, which was derived from a rare liver metastasis that arose in the third cycle of pancreas injection of Colo357FG cells (15). These results show that TrkB was overexpressed in the highly metastatic Colo357L3.6pl cells but not in nonmetastatic Colo357FG cells or less metastatic Colo357L3.3pl cells, and some of the pancreatic cancer samples, suggesting that the overexpression of TrkB may be involved in pancreatic cancer invasion and metastasis. Furthermore, both

TrkA and TrkC, the other members of Trk family of receptor tyrosine kinases, were also overexpressed in Colo357L3.6pl, but not in Colo357FG cells (Fig. 1D), suggesting a possibility that these members of Trk family of receptor tyrosine kinases are also involved in pancreatic cancer invasion and metastasis.

TrkB Is Overexpressed in Human Pancreatic Adenocarcinoma and Correlated with Liver Recurrence

To determine TrkB protein expression in human pancreatic adenocarcinoma, paraffin sections from 54 surgically treated pancreatic adenocarcinoma cases and five normal pancreatic tissue samples were subjected to immunohistochemical analysis with affinity-purified anti-human TrkB antibody. Specific TrkB staining was detected in 50% (27 of 54) of the adenocarcinoma samples, but no staining was detected in the normal controls (Fig. 2A-D). No nonspecific binding or cross-reactivity of this antibody to other proteins was detected by Western blot analysis (Fig. 1E). These results suggest a possible role of TrkB overexpression that contributes to the metastatic potential of human adenocarcinoma. In addition, TrkB immunoreactivity was much weaker and diffuse in the nerve cells of the tumor specimens (Fig. 2E). TrkB immunoreactivity was also observed in Islet cells (Fig. 2F), which are consistent with several earlier findings (38, 39).

To explore whether TrkB overexpression in the patient samples was associated with clinical outcome, we compared TrkB expression and clinical data from the 54 surgically treated pancreatic cancer cases. Annotated clinical data on the 54 patients was available from the pancreatic cancer surgical database. Univariate analysis correlating TrkB immunostaining to clinical outcome showed a significant association with perineural invasion ($P = 0.026$) and positive retroperitoneal margin ($P = 0.0005$, Table 1). Although there was not a strong

Immunohistochemical analysis of TrkB in pancreatic adenocarcinoma

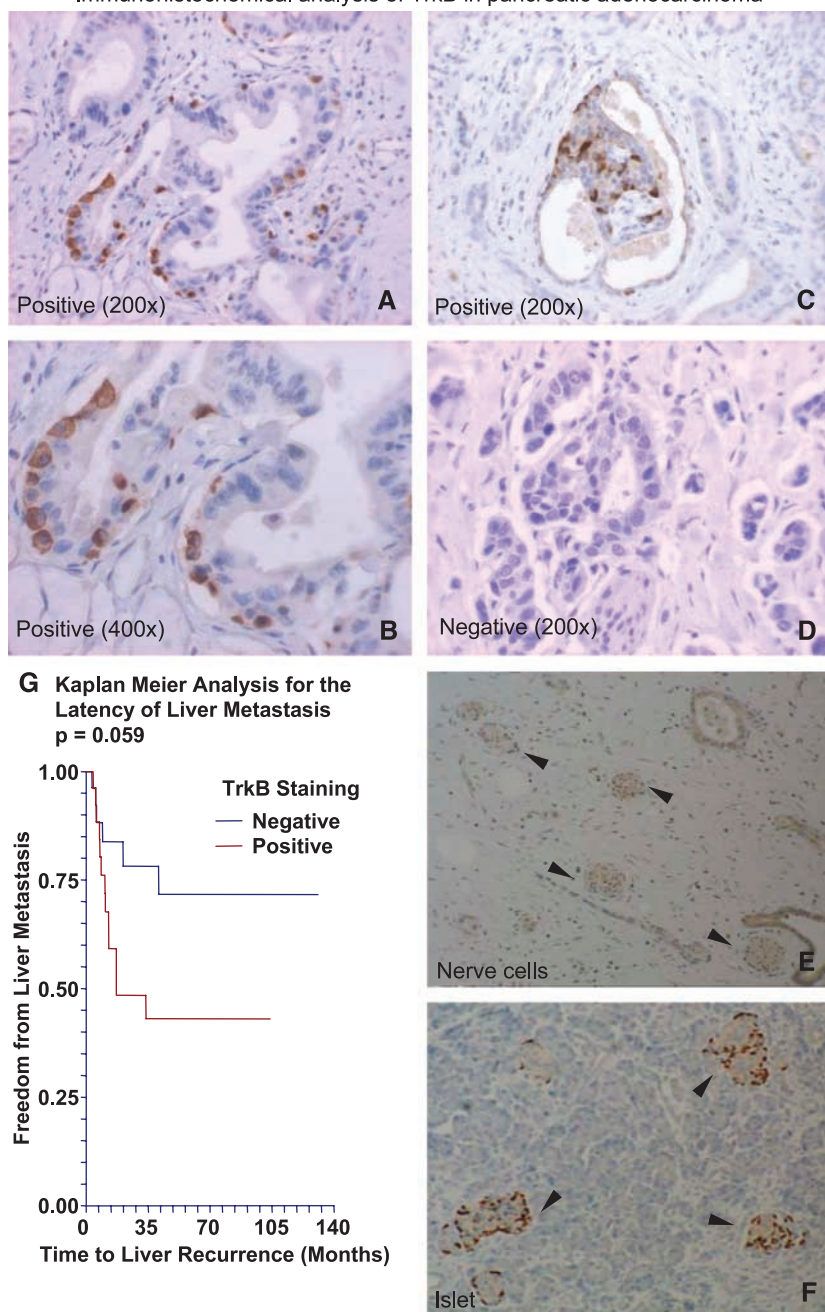


Fig. 2 Immunohistochemical staining for TrkB expression in human pancreatic adenocarcinoma tissue samples. In *A* and *B*, overexpression of TrkB in the same section of pancreatic cancer specimen with different magnification. *C*, overexpression of TrkB in different pancreatic cancer specimen. *D*, pancreatic cancer without detectable TrkB expression. *E*, weak and diffused TrkB immunostaining was observed in nerve cells in the pancreatic tumor specimens. *F*, strong TrkB immunoreactivity was observed in Islet cells. *G*, correlation between TrkB expression in tissue samples from pancreatic cancer patients and time to liver recurrence, analyzed by using Kaplan-Meier statistics.

association between TrkB expression and disease-free survival, overall survival or absence of recurrence as shown by the different Cox regression models (Table 2), lack of TrkB expression was significantly correlated with freedom from liver metastasis (hazard ratio, 0.3; 95% confidence interval, 0.1-0.8; $P = 0.01$). A similar association was found in the Kaplan-Meier analysis for time to liver recurrence (Fig. 2G). Liver metastasis occurred significantly earlier in patients with TrkB-positive tumors than in patients with negative tumors. Taken together, these results suggest that patients with TrkB-expressing tumors were at higher risk for developing early liver recurrences,

supporting the notion that the overexpression of TrkB may be involved in pancreatic cancer invasion and metastasis.

Constitutive Activation of the Extracellular Signal-Regulated Kinase-Mitogen-Activated Protein Kinase Pathway and AP-1 and its Downstream Target Genes *IL-8* and *VEGF* in Colo357L3.6pl Cells

To determine whether TrkB kinase activity was elevated due to increased TrkB expression and whether additional intracellular signaling pathways were altered between Colo357L3.6pl and Colo357FG cells, TrkB *in vitro* kinase assay, Western and

Table 1 Correlation between clinical outcome and TrkB immunostaining

Variable	Category	Cases	TrkB staining		<i>P</i> (χ^2)	Odds ratio (95% confidence interval)
			Negative	Positive		
Gender	Male	39	18	21	0.36	1.8 (0.5-5.9)
	Female	15	9	6		
Age	<65	28	14	14	1.0	1.0 (0.3-2.9)
	>65	26	13	13		
pT	T1	17	7	10	0.38	2.5 (0.8-7.5)
	T2 or T3	37	20	17		
pN	Negative	24	9	15	0.10	4.2 (1.4-12.1)
	Positive	30	18	12		
pTNM	I or II	21	8	13	0.17	2.2 (0.7-6.7)
	III or IV	33	19	14		
Perineural invasion	Negative	13	10	3	0.026	0.2 (0.1-0.9)
	Positive	41	17	24		
Retroperitoneal margin	Negative	41	26	15	0.0005	0.05 (0-0.4)
	Positive	13	1	12		

Northern blot, electrophoretic mobility shift assay, reporter gene assay, and RNase protection assays were done. As shown in Fig. 3A, Colo357L3.6pl cells had higher level of TrkB kinase activity and Erk-1 phosphorylation than did Colo357FG cells. This finding seems to be independent of epidermal growth factor receptor expression as there was no difference in the amount of total and activated (phosphorylated) epidermal growth factor receptor in the two cell lines (data not shown). Fig. 3B shows that Elk-1 and AP-1 DNA binding activities were higher in Colo357L3.6pl cells than in Colo357FG cells. These findings are consistent with the higher *c-fos* mRNA expression in Colo357L3.6pl cells (Fig. 3C). Furthermore, expression of the AP-1 downstream target genes *IL-8* and *VEGF* was higher in Colo357L3.6pl cells (Fig. 3C). Western blot analysis showed that expression of VEGF was greatly induced in the media conditioned by Colo357L3.6pl cells, but not by Colo357FG cells (Fig. 3D). To determine a possible role of TrkB in the regulation of AP-1 activity, we analyzed AP-1 reporter gene activity in Colo357L3.6pl and Colo357FG cells transiently transfected with a plasmid containing a luciferase gene regulated by wild-type or mutant AP-1 enhancer elements. As shown in Fig. 3E, AP-1-mediated transcriptional activity was significantly higher in Colo357L3.6pl cells than in Colo357FG cells and was significantly reduced in both cell lines by a mutant AP-1 reporter gene construct. Taken together, these results suggest that TrkB may be involved in mitogen-activated protein kinase and AP-1 activation, thereby up-regulating important AP-1 downstream target genes such as *IL-8* and *VEGF* to promote invasion and metastasis.

Overexpression of the Proapoptotic Molecules bik and FAS by Colo357FG Cells

The results from the cDNA microarray analysis of Colo357L3.6pl and Colo357FG cells also suggest that the expression of an IAP-related gene was down-regulated in Colo357L3.6pl (Fig. 1A and B). This finding was consistent with a role for TrkB in maintaining the survival or preventing apoptosis (40–42). To identify the differential expression of genes involved in apoptosis, but not included in this microarray, RNA was isolated from both Colo357FG and Colo357L3.6pl cells and analyzed with an RNase protection assay (Fig. 3F and G). Expression of the proapoptotic molecule *bik* was nearly nine times higher in the parental cell line Colo357FG than in the highly metastatic cell line Colo357L3.6pl (Fig. 3H) and FAS mRNA expression was more than thrice higher in Colo357FG cells (Fig. 3J). Furthermore, increased Bcl-2 expression in L3.6pl cells correlated with apoptotic resistance and metastatic potential and Bcl-2 overexpression may be involved in the metastatic progression of pancreatic carcinoma (43). There was no significant difference in the expression of other apoptosis-related proteins, including *bcl-xS*, *bak*, *bax*, *mcl-1*, caspase 8, *fas*-associated death domain protein, tumor necrosis factor receptor-associated death domain protein, tumor necrosis factor-related apoptosis-inducing ligand, FAS-associated factor, and tumor necrosis factor receptor p55 (Fig. 3H and J). The results suggest that increased expression of *bcl-2* and decreased expression of *bik* and FAS may contribute to the resistance to apoptosis, thereby promoting the invasive and metastatic potential in the Colo357L3.6pl cells.

Table 2 Cox proportional hazard ratios for patients with TrkB-positive staining tumors using different Cox regression models

End point	Univariate, HR (95% CI) <i>P</i>	Reduced model (four variables)* HR (95% CI) <i>P</i>	Fuller model (six variables)† HR (95% CI) <i>P</i>
Disease-free survival	0.7 (0.3, 1.3) 0.21	0.5 (0.2, 1.0) 0.050	0.8 (0.4, 1.6) 0.47
Freedom from liver metastasis	0.4 (0.2, 1.1) 0.056	0.3 (0.1, 0.8) 0.010	0.4 (0.1, 1.2) 0.080
Overall survival	0.6 (0.3, 1.2) 0.13	0.4 (0.2, 0.9) 0.032	0.7 (0.3, 1.5) 0.33
Freedom from recurrence	0.6 (0.3, 1.3) 0.19	0.5 (0.2, 1.0) 0.037	0.7 (0.3, 1.6) 0.39

Abbreviation: HR, hazard ratio; CI, confidence interval.

*Independent variables: age, sex, tumor stage, and TrkB staining.

†Independent variables: age, sex, tumor stage, TrkB staining, perineural invasion, and retroperitoneal margin.

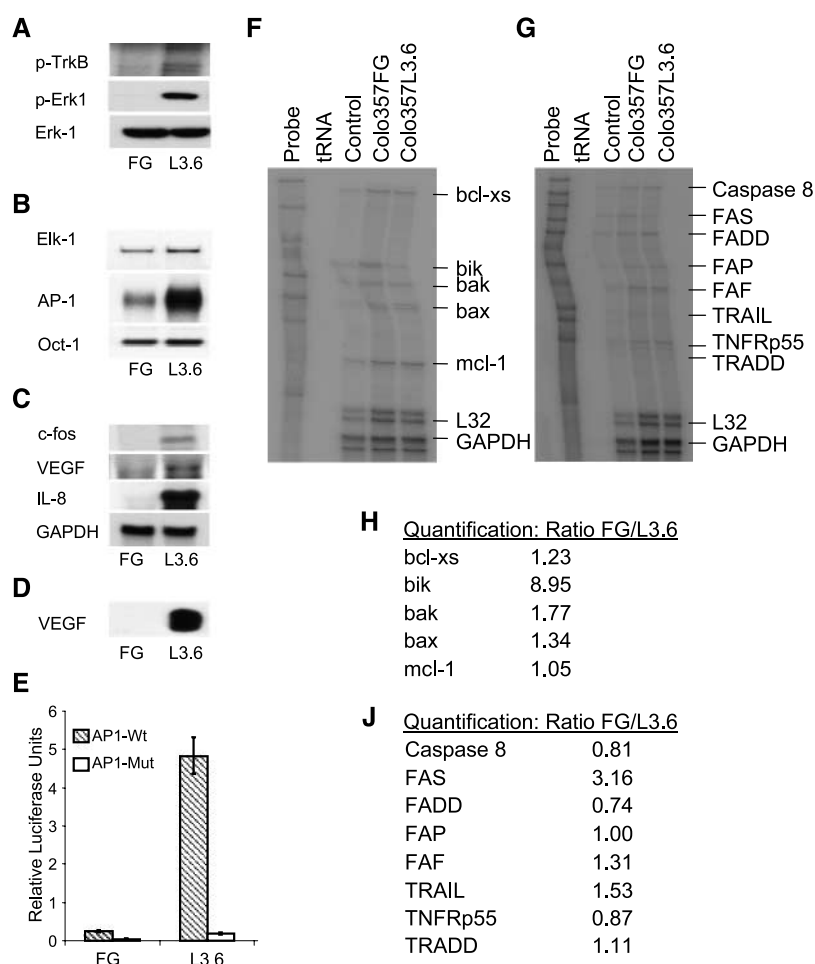


Fig. 3 Activation of ERK-MAPK and AP-1 and overexpression of VEGF and IL-8 in Colo357L3.6pl cells. *A*, analysis of TrkB kinase activity and Erk-1 phosphorylation in Colo357FG (FG) and Colo357L3.6pl (L3.6) using TrkB *in vitro* kinase assay and an anti-phospho-Erk1 antibody. *B*, electrophoretic mobility shift assays of DNA-binding activity for Elk-1 and AP-1 in Colo357FG and Colo357L3.6pl cells. Oct-1 DNA binding activity was measured as a loading control. *C*, Northern blot analysis of *c-fos* and the AP-1 downstream target genes *VEGF* and *IL-8* in Colo357FG and Colo357L3.6pl cells (GAPDH served as a loading control). *D*, Western blot analysis of VEGF expression in conditioned medium from Colo357FG and Colo357L3.6pl cells. *E*, Luciferase reporter gene assay for AP-1 activity in Colo357FG and Colo357L3.6pl cells. The luciferase activities were normalized to the *Renilla* luciferase activity of the internal control as described in Material and Methods. *F-J*, Analysis of expression of proapoptotic and antiapoptotic genes in Colo357FG and Colo357L3.6pl cells. RNA was isolated from Colo357FG and Colo357L3.6pl cells and ribonuclease protection assays were carried out using the Riboquant Multiprobe Protection Assay System to determine proapoptotic and antiapoptotic gene expression. The quantification was obtained using phosphoimage analysis.

TrkB and VEGF Overexpression in Colo357L3.6pl-Derived Tumors and Metastases in an Orthotopic Nude Mouse Model

To determine the expression of TrkB, VEGF, and IL-8 in an *in vivo* model, we orthotopically injected nude mice with Colo357FG and Colo357L3.6pl cells, harvested the resulting Colo357FG and Colo357L3.6pl primary pancreatic tumors and the Colo357L3.6pl liver metastases, and subjected them to immunohistochemical analysis for TrkB, VEGF, and IL-8 (Fig. 4A-C). Colo357FG tumors expressed TrkB, VEGF, and IL-8 only weakly, but Colo357L3.6pl primary tumors showed much greater expression of these three proteins. Even stronger TrkB, VEGF, and IL-8 expression was found in liver metastases from Colo357L3.6pl cells. These results summarized in Fig. 4D support the findings shown in Figs. 1, 2, and 3. Taken together, these results suggest a possible role of TrkB overexpression in the highly metastatic potential of Colo357L3.6pl cells.

DISCUSSION

Our results showed that the highly metastatic pancreas cancer cell line Colo357L3.6pl overexpressed TrkB, relative to its parental cell line Colo357FG, using cDNA microarray analysis. The overexpression of TrkB was further shown by reverse transcription-PCR and Western blot analysis. Overexpression of TrkA, and TrkC members of Trk family of receptor

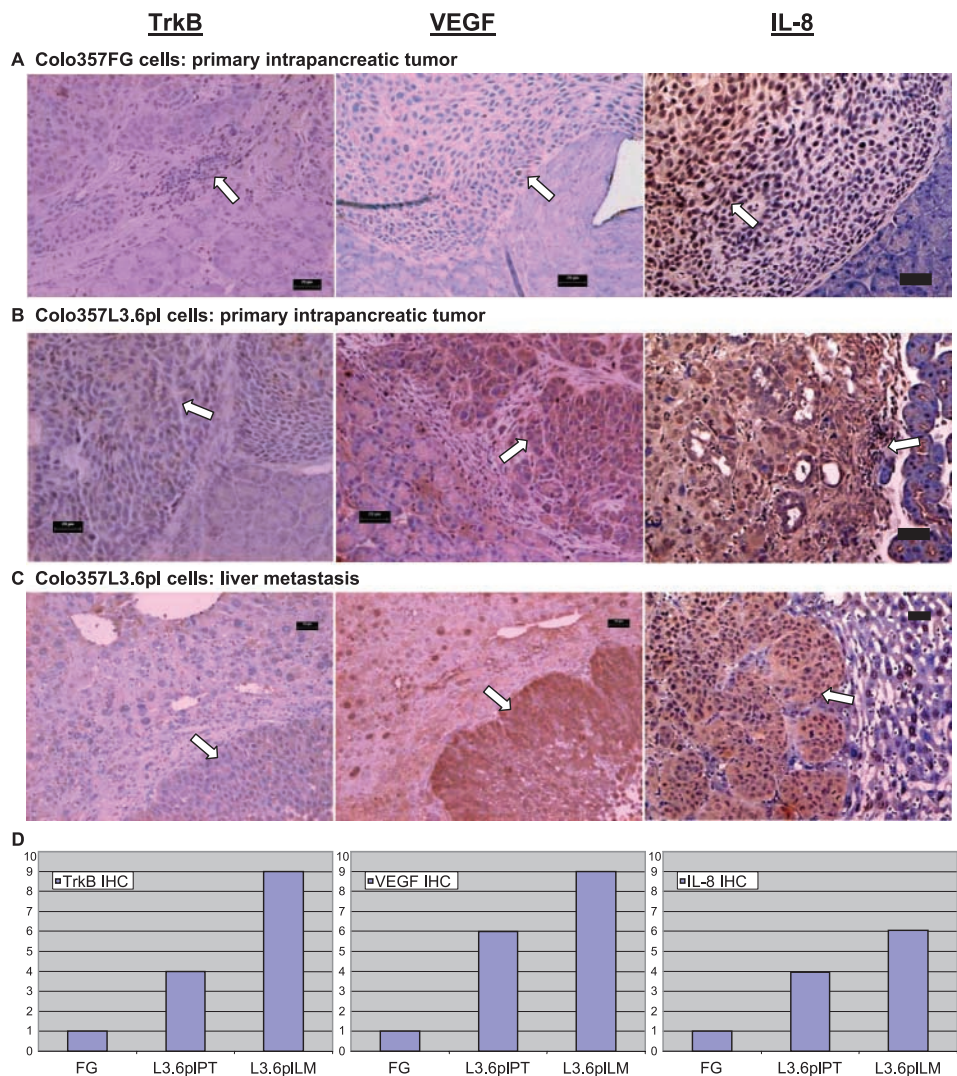
tyrosine kinases were also detected in Colo357L3.6pl, but not in Colo357FG cells and only overexpression of TrkA was found in a less metastatic Colo357L3.3pl cell line (Fig. 2). Differential activation of ERK-1 kinase and transcription factor AP-1 and the differential TrkB, VEGF, and IL-8 expression were consistently found in Colo357L3.6pl and Colo357 FG. The cDNA microarray analysis of Colo357L3.6pl and Colo357FG cells also suggested that the expression of an IAP-related gene was down-regulated in Colo357L3.6pl (Fig. 1). Additional analyses using Western and Northern blot analyses and the multiple probe RNA protection assay were done to identify some of the genes potentially involved in cancer metastasis that were not included in the cDNA microarray. These results also implicated the overexpression of VEGF and IL-8 and down regulation of the expression of proapoptotic *bik* and *FAS* genes in the highly metastatic cell line Colo357L3.6pl (Fig. 3). It is possible that activation of ERK-1 and AP-1 and overexpression of IL-8, VEGF, and Bcl-2 and down-regulation of *bik* and *FAS* might be induced by a combination of many genetic alterations such as several growth factor receptors including Trk and fibroblast growth factor receptor (27, 43). Taken together, our results suggest that alterations in the expression of TrkB, VEGF, IL-8, *bik*, and *FAS* may be involved in pancreatic cancer invasion and metastasis. These results are consistent with previous reports demonstrating that expression of various neurotrophin ligands

and their cognate trk receptors in metastatic prostate cancer cells results in activation of an autocrine survival pathway (44, 45).

Recently a progression model for pancreatic cancer has been proposed based on the unique profile of genetic and molecular alterations in pancreatic cancer (46, 47). Overexpression of HER-2/*neu* occurs in about 90% of duct lesions and in 25% of invasive pancreatic adenocarcinomas, point mutations in the *K-ras* gene in 45% of duct lesions and in about 80% to 95% of pancreatic adenocarcinomas, inactivation of the *p16* gene in 71% of ductal lesions, inactivation of p53 in 50% to 75% of pancreas cancers, and inactivation of DPC4 in 50% of the pancreatic cancers (46, 47). In this study, we showed overexpression of TrkB in 50% of the human pancreatic cancer specimens tested. Weak and diffuse TrkB immunoreactivity was observed in the nerve cells of the tumor specimens (Fig. 2E). TrkB immunoreactivity was also observed in Islet cells (Fig. 2F), which are consistent with several earlier findings that the Trk has roles in both ontogenesis and oncogenesis in different cell types by using different Trk family receptors and intracellular signaling

pathways. Using reverse transcription-PCR analysis of laser-captured cells, Ketterer et al. had the similar observation and suggested potential paracrine and autocrine actions of neurotrophins in pancreatic cancer. They also pointed out that the Trk receptor mRNA and protein levels were not concordant, raising the possibility that there are various factors that control the post-translational regulations of TrkB receptor in different cell types. Furthermore, we found a correlation between TrkB overexpression and perineural invasion, positive retroperitoneal margin, and liver metastatic latency in this exploratory study. Our results suggest that overexpression of Trk family receptor tyrosine kinases may play an important role in pancreatic cancer invasion and metastasis. However, the underlying molecular mechanism by which overexpression of Trk family receptor tyrosine kinase is induced remains unclear. The overexpression of TrkA, TrkB, and TrkC detected in these pancreatic cancer cell lines may imply that these three members of receptor tyrosine kinase might be coregulated by the similar but yet unidentified transcription factors. To provide a better understanding of the molecular basis of

Fig. 4 Overexpression of TrkB, VEGF, and IL-8 in pancreatic tumors from nude mice. Immunohistochemical staining for TrkB, VEGF, IL-8 expression in primary intrapancreatic tumor specimens derived from (A) Colo357FG cells, (B) Colo357L3.6pl cells, (C) liver metastasis from Colo357L3.6pl cells, and (D) quantification of the immunostainings. Bar, 20 μ m.



pancreatic cancer metastasis, it is very important to identify signaling regulators that induce overexpression of Trk family receptor tyrosine kinases.

Using a nude mouse xenograft model, it has been shown that the selective Trk receptor tyrosine kinase inhibitor CEP-701 reduced the size of tumors derived from a nonmetastatic pancreatic cancer cell line Panc-1 in comparison with vehicle-treated controls (48). These results suggest that the Trk signaling pathways may be an important new target for the treatment of pancreatic cancer. However, it is still unclear whether Trk receptor blockade will decrease the incidence of metastasis in Colo357L3.6pl. It is very interesting to determine a possible link between the overexpression of Trk receptor tyrosine kinases and pancreatic cancer invasion and metastasis using CEP-701 and its related inhibitors when they become available.

In summary, our results revealed that TrkB may play an important role in perineural invasion and metastasis in adenocarcinoma of the pancreas, suggesting a role for neurotrophin receptors in perineural invasion by facilitating tumor cell growth through inhibition of apoptosis and promotion of angiogenesis. This study also shows the utility of gene expression profiling in the identification of prognostic molecular tumor markers, which may help define subpopulations at high risk of tumor progression and thus may influence therapeutic considerations.

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