The G691S RET Polymorphism Increases Glial Cell Line–Derived Neurotrophic Factor–Induced Pancreatic Cancer Cell Invasion by Amplifying Mitogen-Activated Protein Kinase Signaling

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Abstract

Mutations of the RET proto-oncogene are responsible for several inherited human diseases and may function as genetic modifiers of the disease. However, the role of RET mutations in pancreatic cancer has not been studied. Expression of the glial cell line–derived neurotrophic factor (GDNF) receptors RET and GDNF family receptor α1 (GFRα1) in human pancreatic cancer cells was determined by Western blot, immunofluorescence, and flow cytometry. The effect of GDNF on cell proliferation and invasion was assessed. Small interfering RNA and antibodies were used to evaluate the involvement of RET. The G691S RET polymorphism was analyzed by sequencing and restriction analysis. The modifying effect of G691S RET on GDNF-induced invasion and mitogen-activated protein kinase (MAPK) signaling was evaluated. Transfection studies with wild-type and mutated RET determined the functional role of the G691S polymorphism. Pancreatic cancer specimens and matched tissues were analyzed for the presence of the G691S RET polymorphism. GDNF receptors were found on all cell lines. GDNF increased pancreatic cancer cell proliferation and invasion, which was mediated by RET. The effect of GDNF was more profound in cells with the G691S RET polymorphism ($P < 0.01$). G691S RET correlated with an enhanced activation of the downstream extracellular signal-regulated kinase pathway. Overexpression of G691S RET increased pancreatic cancer cell invasion. The G691S RET polymorphism was also detected in human pancreatic tumors and represented a somatic mutation in some patients. These findings indicate that the G691S RET single nucleotide polymorphism may directly correlate with the aggressive growth of pancreatic cancers and may function as a genetic modifier or even low-penetrance gene. (Cancer Res 2005; 65(24): 11536-44)

Introduction

Pancreatic ductal adenocarcinoma is an extremely lethal disease, which is characterized by its propensity to infiltrate adjacent tissues and to metastasize even at early stages (1). Absence of specific symptoms, lack of early detection markers, aggressive tumor growth, and resistance to conventional chemotherapy and radiotherapy regimens conspire to culminate in a median overall survival of <9 months and annual mortality figures virtually equaling incidence numbers (2). A characteristic pattern of genetic alterations in pancreatic cancer has been elucidated, which include activating mutations in oncogenes, e.g., *KRAS*, and loss-of-function mutations in tumor suppressor genes, e.g., *INK4A* and *TP53*. The genetic changes seem to temporally correlate with the development of pancreatic intraepithelial neoplasias, precursor lesions of pancreatic adenocarcinomas (1, 3). However, it is still not well understood how these signature genetic lesions contribute to the biological characteristics of this disease. In addition to alterations in oncogenes and tumor suppressor genes, amplified autocrine and paracrine growth factor signaling loops, e.g., the epidermal growth factor pathway, evidently contribute to the aggressive growth pattern of pancreatic cancers (4). Germline genetic variants of these growth factors and their cognate receptors are now being increasingly recognized as critical modulators of cancer biology (5).

Neurotrophic factors, which include neurotrophins, neurokines, and glial cell line–derived neurotrophic factor (GDNF) family ligands (GFL), regulate many critical aspects of the ontogeny of neurons. GDNF, which was purified and initially characterized as a growth factor promoting the survival of dopaminergic neurons in the midbrain (6), supports several neuronal populations in the central and peripheral nervous system. Outside the nervous system, GDNF functions as a morphogen in kidney development and regulates spermatogonial differentiation (7). In pancreatic cancers, increased levels of neurotrophic factors have recently been described (8) and correlated to proliferation and invasion (9). Detailed analysis suggests that neurotrophic factors are mainly secreted from intrapancreatic and extrapancreatic nerves and bind to their cognate receptors on cancer cells (8, 9). A recent study showed that GDNF is detectable in intrapancreatic nerves in normal pancreas, but is strongly expressed in pancreatic cancers together with the GDNF receptor RET, which correlated to invasion and survival after surgical resection (10).

The *RET* (*re*arranged during *t*ransfection) gene was identified as a novel oncogene activated by DNA rearrangement in 1985 (11) and encodes a receptor tyrosine kinase with four cadherin-related motifs and a cysteine-rich region in the extracellular domain (12). RET is essential for the normal development of the sympathetic, parasympathetic, and enteric nervous systems. The RET protein is a subunit of a multimolecular complex that binds growth factors of the GDNF family. GFLs bind RET in conjunction with glycosylphosphatidylinositol-anchored coreceptors, designated GFRα. The GFL-GFRα complex brings together two RET molecules, thereby triggering transphosphorylation of specific tyrosine residues in their kinase domains and several intracellular signaling pathways

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-05-2843

(7, 13). Various germline point mutations in the extracellular cysteine-rich and intracellular tyrosine kinase domains of the RET proto-oncogene have been shown to be responsible for multiple endocrine neoplasia 2 (MEN 2), an inherited cancer syndrome characterized by the development of medullary thyroid carcinomas (14–17). Somatic RET point mutations and gene deletions are found in 40% to 50% of sporadic medullary thyroid cancers (18) and rearrangement of the *RET* gene is one of the most common genetic alterations identified in thyroid papillary carcinomas (19). Besides oncogenic mutations, several *RET* variants are considered low-penetrance genes or genetic modifiers in MEN 2 patients (20). In contrast to the extensive literature on RET and the thyroid gland, there is no report describing the functional role of RET or its variants in pancreatic cancer.

In this study, we describe the occurrence of a RET receptor polymorphism in human pancreatic cancers. Furthermore, we provide evidence that the G691S RET polymorphism amplifies the proinvasive and proliferative properties of GDNF on pancreatic cancer cells by enhancing mitogen-activated protein kinase (MAPK) signaling. To our knowledge, this is the first report describing the functional effects of the G691S RET polymorphism on a cellular and molecular level in pancreatic cancer or other cancers.

Materials and Methods

Reagents

Human recombinant GDNF was purchased from Chemicon International (Temecula, CA). The MAP/extracellular signal-regulated kinase (ERK) kinase-1 inhibitor PD98059 and the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin were obtained from Cell Signaling Technology (Beverly, MA). The following antibodies were used: rabbit polyclonal anti-RET (C-19) and rabbit polyclonal anti-GFRα1 (H-70) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-β-actin from Sigma Chemical Co. (St. Louis, MO); mouse monoclonal anti-phosphotyrosine antibody (4G10) from Upstate (Lake Placid, NY); and rabbit polyclonal antibodies against phospho-ERK1/2, total ERK1/2, phospho-AKT, and total AKT from Cell Signaling Technology. The full-length human RET expression vector [Rc/cytomegalovirus (CMV)-RET] was a generous gift from M. Takahashi (Nagoya University Graduate School of Medicine, Nagoya, Japan).

Cell Culture

The human pancreatic cancer cell lines AsPC-1 (well to poorly differentiated), BxPC-3 (well to poorly differentiated), Capan-2 (well differentiated), MIA PaCa-2 (undifferentiated), and PANC-1 (poorly differentiated), as well as the murine NIH/3T3 fibroblasts, were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described previously (21, 22).

Expression of RET and Glial Cell Line–Derived Neurotrophic Factor Family Receptor α1

Western blot analysis. Total cell lysates from confluent cultures were prepared using ice-cold modified radioimmunoprecipitation assay buffer (RIPA) containing leupeptin, aprotinin, and pepstatin A. Aliquots were fractionated on 8% SDS-PAGE and transferred to nitrocellulose membranes. RET and GFRα1 proteins were detected using rabbit anti-RET and anti-GFRα1 antibodies and a horseradish peroxidase–conjugated anti-rabbit immunoglobulin as a secondary antibody (Pierce, Rockford, IL). Protein-antibody complexes were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunofluorescence. Pancreatic cancer cells were seeded on four-chamber culture slides (BD Biosciences Discovery Labware, Bedford, MA) and grown to confluence. Cells were fixed with −20°C methanol, blocked with 5% goat serum for 20 minutes, and followed by incubation with rabbit anti-RET antibody or rabbit anti-GFRα1 antibody (7.5 μg/mL in 1.5% goat serum) for 2 hours at room temperature. Slides were then incubated with fluorochrome-conjugated goat anti-rabbit IgG (Chemicon International) or Texas-red–conjugated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:1,000 for 1 hour in the dark. After mounting in VECTASHIELD Mounting Medium (Vector Laboratories) images were captured using an Olympus BX60 fluorescence microscope. Isotype-matched antibodies were used as negative controls using the same staining and image-capturing protocol.

Flow cytometry. Cells were detached using 0.2% EDTA/PBS, washed with 0.2% bovine serum albumin/0.1% sodium azide in PBS and stained with anti-RET (2 μg/mL) or anti-GFRα1 (2 μg/mL) antibodies for 30 minutes at 4°C, followed by fluorochrome-labeled secondary antibodies (2 μg/mL) for 20 minutes at 4°C. Fluorescent cells were analyzed by FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Isotype-matched immunoglobulins were used as negative controls.

Cell Proliferation Assay

Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and cell counting as described previously (23, 24). Briefly, pancreatic cancer cells were incubated with GDNF (1, 10, and 100 ng/mL) for 24 hours in serum-free medium. In some experiments, cells were preincubated with the MEK-1 inhibitor PD98059 (10 μmol/L) or the PI3K inhibitor wortmannin (200 nmol/L) for 30 minutes before adding GDNF. Cell proliferation was determined by measuring the absorbance after adding MTT or by counting the cells under a light microscope. Experiments were done at least in triplicate using separate cultures.

Invasion Assay

Invasion assays were done using a Matrigel invasion chamber (BD Biosciences Discovery Labware) as described previously (25). Briefly, pancreatic cancer cells were seeded in the upper chamber in serum-free media in the presence of GDNF (10 and 100 ng/mL). In some experiments, cells were preincubated with the MEK-1 inhibitor PD98059 (10 μmol/L), the PI3K inhibitor wortmannin (200 nmol/L), or anti RET antibodies (1 μg/mL) for 30 minutes before adding GDNF. In small interfering RNA (siRNA) studies, cells were transfected with RET siRNA 48 hours before being seeded into the upper chamber. Complete medium containing 20% fetal bovine serum (FBS) served as a chemoattractant in the lower chamber. GDNF was added to the lower compartment at the same concentration as in the upper chamber. After 24 hours, invading cells on the lower membrane surface were stained and counted. All invasion experiments were done at least in triplicate using separate cultures and the same lot of Matrigel chambers.

RNA Interference

siRNAs specifically targeting RET exon 2 (5′-GGGAUGCUUACUGGGA-GAAtt-3′) were custom-designed by Ambion, Inc. (Austin, TX). Cells were transfected with RET siRNA using the Silencer Transfection kit (Ambion). Briefly, cells were grown in 35 mm dishes and overlaid with the transfection mixture containing siRNA (20 nmol/L) and siPORT Lipid (Ambion) in Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA). After 4-hour incubation, complete medium with 10% FBS was added and cells were incubated for another 2 days. Scrambled siRNA served as a negative control.

G691S RET Polymorphism in Cultured Cells

RET coding regions were amplified using genomic DNA of pancreatic cancer cells as a template. The primer pair flanking exon 11 was designed as follows (Genbank accession number AJ243297): forward (5′-GACACGG-CAGGCTGGAGAGC-3′, 14,872-14,891), reverse (5′-TCCCTCCCTGGAAGG-CAGCT-3′, 15,273-15,292). Direct sequence analysis of the amplified and purified DNA fragment was done using an ABI prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA). For restriction enzyme analysis, the amplified and purified RET exon 11 was digested with *Bsr*I (New England Biolabs, Beverly, CA). Samples were purified and separated on an agarose gel.

Extracellular Signal-Regulated Kinase Phosphorylation

Activation of the ERK1/2 was determined as described previously (25). Briefly, pancreatic cancer cells were incubated in serum-free medium with the indicated ligands and total cell lysates were separated on 8% SDS-PAGE

and transferred to nitrocellulose membranes. Membranes were probed with antibodies recognizing specifically the dually phosphorylated ERK1/2. Detection of total ERK1/2 levels served as loading controls.

Immunoprecipitation

Total cell lysates were prepared using ice-cold modified RIPA buffer and precleared by adding Protein A-agarose bead slurry (Roche Diagnostics, Alameda, CA) at 4°C for 1 hour. RET immunoprecipitating antibodies (2 μg/100 μg protein) were added to the precleared cell lysates overnight at 4°C. Immunocomplexes were captured by adding Protein A-agarose bead slurry for 1 hour at 4°C. The agarose beads were washed with ice-cold modified RIPA buffer and boiled in electrophoresis buffer. After centrifugation, the remaining supernatant was fractionated on 8% SDS-PAGE and transferred to nitrocellulose membranes.

Construction of the G691S RET Plasmid

The G691S RET polymorphism was introduced into the Rc/CMV-RET plasmid by primer-mediated, site-directed mutagenesis using the Quick Change Mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. Details are provided in the Supplementary Data.

RET Receptor Transfection

Cells were transfected as described previously (21, 22). Briefly, pancreatic cancer cells or fibroblasts were grown on 35 mm dishes to 50% confluence and transfected with Rc/CMV-RET (WT-RET) or Rc/CMV-G691S RET (G691S RET) using the LipofectAMINE Plus reagent (Invitrogen) for 48 hours. An empty vector was used as a control.

G691S RET Polymorphism in Human Pancreatic Cancers

PCR. Formalin-fixed paraffin-embedded primary pancreatic ductal adenocarcinomas and matched benign pancreas from 52 patients who underwent surgery at the Department of Surgery, David Geffen School of Medicine at University of California at Los Angeles, were obtained. The study was approved by the University of California at Los Angeles Institutional Review Board. DNA was extracted from tissue blocks as previously described (26, 27). Detection of the RET polymorphism was done using a peptide nucleic acid PCR assay (28).

Sequencing. All samples with detected G691S RET polymorphism and 10 random negative samples were sequenced to validate peptide nucleic acid PCR results. Details are provided in the Supplementary Data.

RET Immunohistochemistry of Human Pancreatic Cancers

Details are provided in the Supplementary Data.

Statistical Analysis

Data are presented as mean ± SD. Differences in the mean of two samples were analyzed by an unpaired t test. Comparisons of more than two groups were made by a one-way ANOVA with post hoc Holm-Sidak analysis for pairwise comparisons and comparisons versus control. Differences in allelic frequency between primary pancreatic tumors and matched tissues were calculated with the χ^2 analysis of contingency tables. An α value of 0.05 was used to determine significant differences. All statistics were done in SigmaStat 3.1 (Systat Software, Inc.).

Results

Expression of the glial cell line–derived neurotrophic factor receptors RET and glial cell line–derived neurotrophic factor family receptor α1. Six human pancreatic cancer cell lines were initially analyzed for the presence of the GDNF receptors. Using immunoblotting, a short (150 kDa) and long (170 kDa) RET isoform together with the GFRα1 receptor were detected at similar levels on all six cell lines (Fig. 1*A*). Protein expression was confirmed by immunofluorescence for both receptors (Fig. 1*B*). Flow cytometry

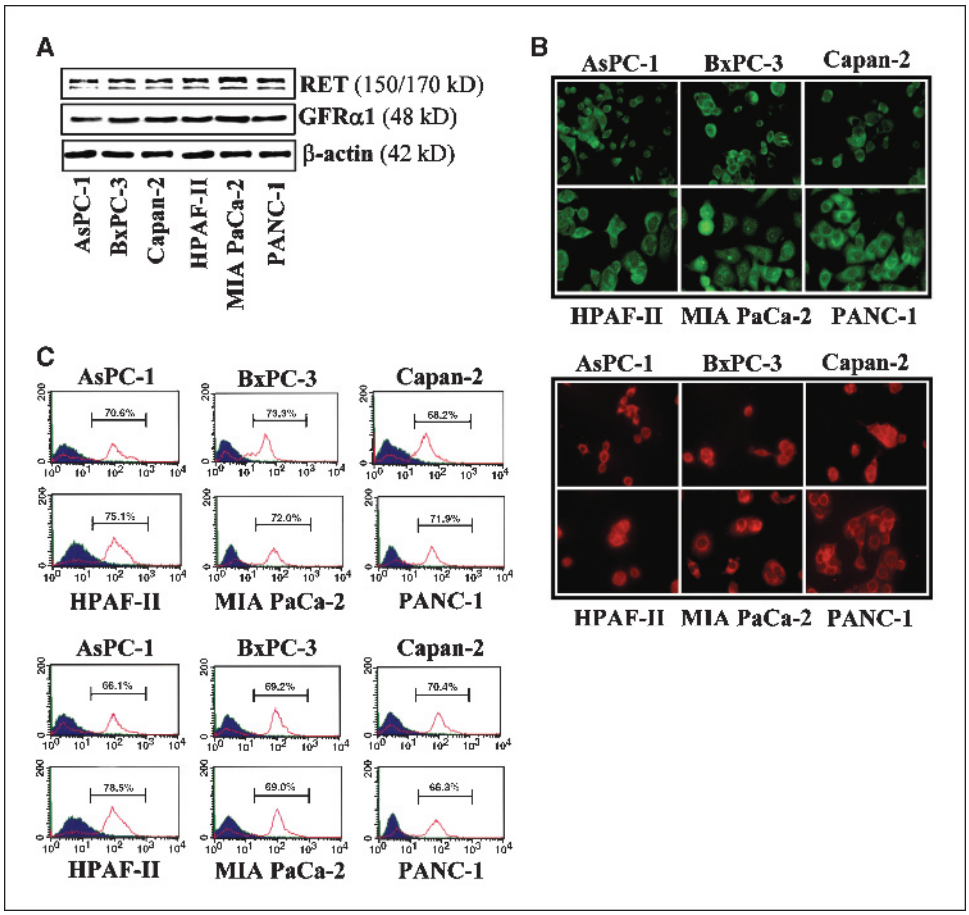

Figure 1. RET and GFRα1 expression in cell lines. *A,* RET and GFRα1 protein expression in pancreatic cancer cell lines was determined in whole cell lysates by immunoblotting. Reprobing with an anti-β-actin antibody served as a control. *B,* RET and GFRα1 protein expression in pancreatic cancer cell lines was confirmed by immunofluorescence. *Top,* pancreatic cancer cells stained with fluorochrome-labeled anti-RET antibodies; *bottom,* staining with Texas red–labeled anti-GFRα1 antibodies. Magnification, ×400. *C,* flow cytometry analysis of RET and GFRα1 protein showed comparable expression levels in all cell lines. Percentage of positively stained cells (*red curve*) compared with control populations (*blue curve*) in which the primary antibody was replaced by an isotype-matched control immunoglobulin at the same concentration. *Top* and *bottom,* flow cytometry analysis with antibodies against RET and GFRα1, respectively.

analysis established comparable expression levels of both receptors in all cell lines. Around 65% to 75% of the entire cell populations stained positive for RET and GFRα1 (Fig. 1*C*) with similar mean fluorescence intensities (not shown).

Glial cell line–derived neurotrophic factor stimulates pancreatic cancer cell proliferation. Having shown expression of RET and GFRα1 on pancreatic cancer cell lines, the effect of GDNF on cell growth was determined. Using the colorimetric MTT proliferation assay, GDNF dose dependently increased proliferation of pancreatic cancer cell lines (Fig. 2*A*). This finding was confirmed by direct counting of cells after 24 hours treatment with GDNF (Fig. 2*B*). Interestingly, whereas GDNF strikingly increased cell growth in Capan-2 and MIA PaCa-2 cells, the effect on AsPC-1 and PANC-1 cells was significantly less prominent ($P < 0.01$; Fig. 2*A* and *B*).

Binding of GDNF/GFRα1 to the RET receptor leads to a coordinated phosphorylation of multiple tyrosine residues in the RET kinase domains, e.g., Tyr^{905}, $Tyr^{1,015}$, $Tyr^{1,062}$, and $Tyr^{1,096}$. Interestingly, $Tyr^{1,062}$ turned out to be the binding site of at least five different docking proteins, SHC, FRS2, DOK4/5, IRS1/2, and Enigma (15). After GDNF-induced binding of SHC to $Tyr^{1,062}$, GRB2/SOS and GAB1/2 are recruited to the receptor complex, leading to activation of the RAS/ERK and PI3K/AKT signaling pathway, respectively (15). The MEK-1 inhibitor PD98059 completely abrogated the GDNF-induced proliferation of MIA PaCa-2 cells, whereas the PI3K inhibitor wortmannin reduced the GDNF-induced cell proliferation only by 30% (Fig. 2*C*). This suggested that the growth stimulating effects of GDNF in pancreatic cancer cells were largely mediated by RET-transduced activation of the RAS/ERK pathway.

Glial cell line–derived neurotrophic factor stimulates cell invasion. GDNF has been shown to function as a migratory signal for peripheral neurons during mammalian embryogenesis (29, 30) and to increase cancer cell invasion (31). We determined whether GDNF modulates pancreatic cancer cell invasion using the Matrigel double-chamber assay. Incubation of pancreatic cancer cells with GDNF in the upper chamber for 24 hours dose-dependently increased invasion of AsPC-1, Capan-2, MIA PaCa-2, and PANC-1 cells (Fig. 3*A* and *B*). The GDNF-induced pancreatic cancer cell invasion was significantly more robust in Capan-2 and MIA PaCa-2 cells ($P < 0.01$; Fig. 3*B*). Preincubation with the MEK-1 inhibitor PD98059 completely blocked GDNF-induced MIA PaCa-2 invasion, whereas the PI3K inhibitor wortmannin had only a partial effect (Fig. 3*C*). GDNF has recently been found to also signal through RET-independent mechanisms (32). To determine whether the GDNF effect was specifically RET receptor-mediated, RET neutralizing antibodies and small interfering RNA (siRNA) were assessed. Preincubation of MIA PaCa-2 cells with RET antibodies but not isotype-matched control antibodies completely inhibited GDNF-induced invasion (Fig. 3*D*). In addition, MIA PaCa-2 cells transfected with RET siRNA had a significantly lower GDNF-induced invasive capacity ($P < 0.01$; Fig. 3*E*). Down-regulation of RET protein expression by siRNA was confirmed by immunoblotting (Fig. 3*F*), which showed over 80% reduction in RET protein levels.

G691S RET polymorphism in cell lines. The finding that GDNF differentially stimulates proliferation and invasion in pancreatic cancer cell lines having comparable levels of the RET receptor prompted us to screen RET for possible polymorphic variants, which may function as genetic modifiers. Single-strand conformation polymorphism analysis of all 20 RET exons has previously

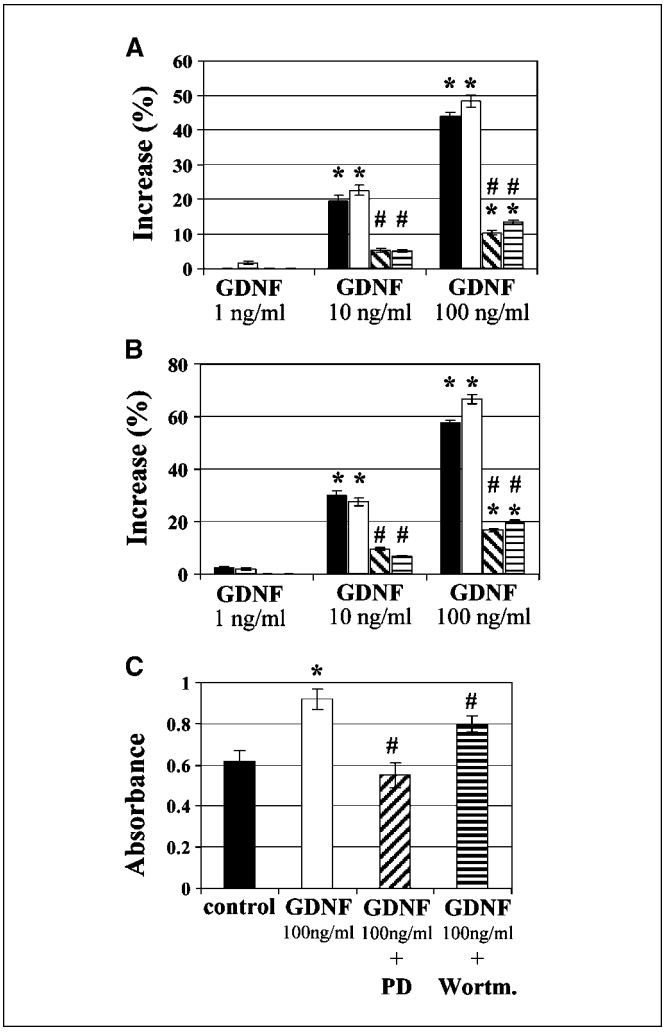

Figure 2. The effect of GDNF on proliferation. Cell proliferation was assessed using the MTT assay (*A*) and cell counting (*B*). Capan-2 (*black columns*), MIA PaCa-2 (*white columns*), AsPC-1 (*diagonal striped columns*), and PANC-1 (*horizontal striped columns*) were treated for 24 hours with GDNF (0-100 ng/mL) in serum-free medium. *Columns,* mean increase in percentage over vehicle-treated cells; *bars,* SD. *, $P < 0.01$ versus control; #, $P < 0.01$ versus Capan-2 and MIA PaCa-2 cells. *C,* the MEK inhibitor PD98059 and the PI3K inhibitor wortmannin inhibit the GDNF-induced increase in MIA PaCa-2 proliferation. MIA PaCa-2 cells were pretreated with PD98059 (*PD*) or wortmannin followed by GDNF for 24 hours in serum-free medium. *Columns,* mean absorbance readings (cell viability); *bars,* SD. *, $P < 0.01$ versus control; #, $P < 0.01$ versus GDNF 100 ng/mL.

documented six polymorphic nucleotide changes, of which only one (GGT→AGT) resulted in an amino acid change (Gly→Ser) at codon 691 in exon 11 (33). Pancreatic cancer cells were first screened for the presence of the G691S RET variant. We found that Capan-2 and MIA PaCa-2 cells are heterozygous for the G691S RET polymorphism. Interestingly, both cell lines that harbor the G691S RET polymorphism responded to GDNF stimulation with a more robust increase in proliferation and invasion than AsPC-1 and PANC-1 cells, which are homozygous for the wild-type allele. The G→A substitution creates a new restriction site for *Bsr*I. A 421 bp fragment of exon 11, containing the nucleotide sequence for codon 691, was amplified by PCR and digested with *Bsr*I. Restriction enzyme analysis confirmed that Capan-2 and MIA PaCa-2 are heterozygous for the *G691S RET* allele, whereas AsPC-1 and PANC-1 cells are homozygous for the wild-type allele (Fig. 4).

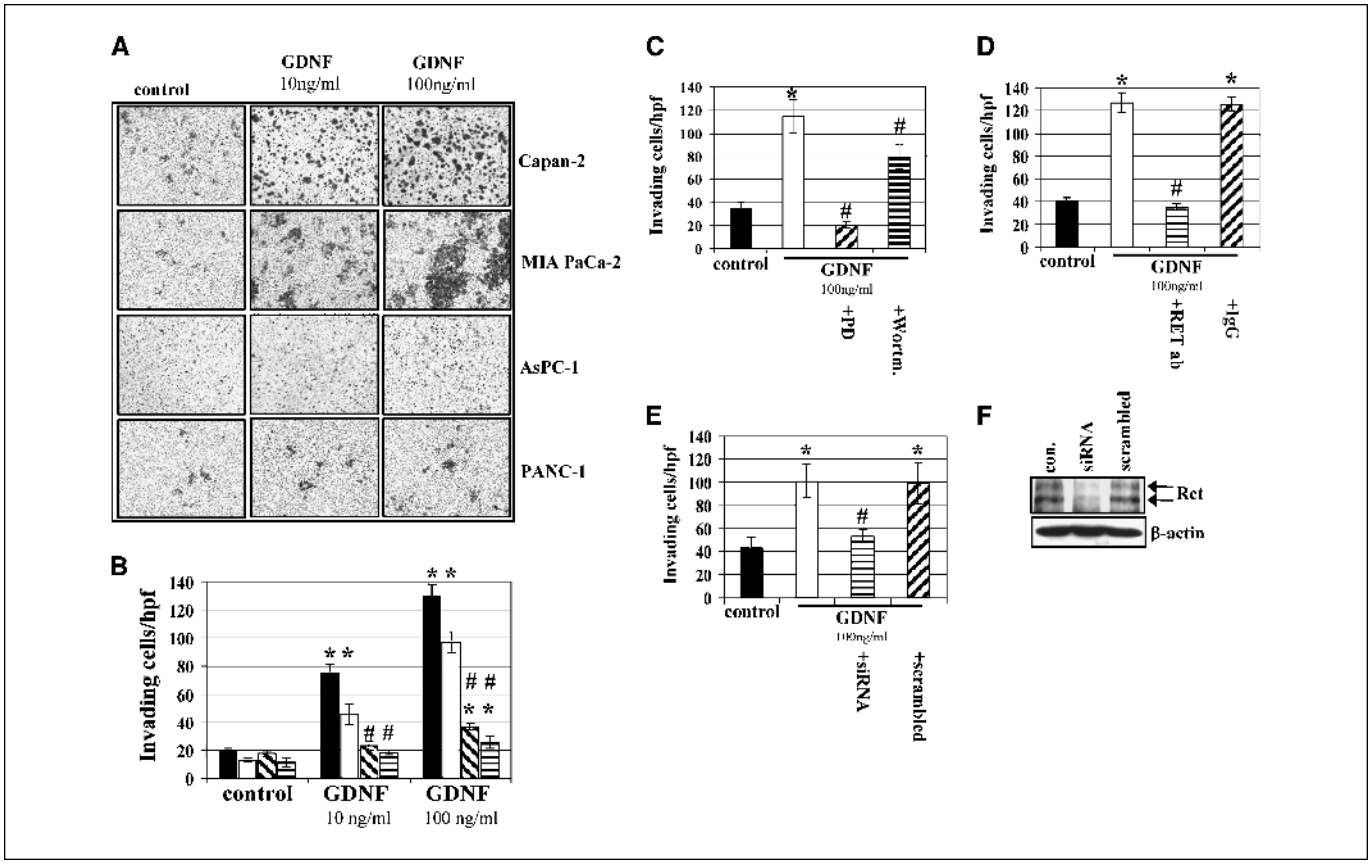

Figure 3. The effect of GDNF on invasion. *A* and *B,* GDNF dose-dependently stimulates pancreatic cancer cell invasion in the Matrigel invasion assay. Capan-2 (*black columns*), MIA PaCa-2 (*white columns*), AsPC-1 (*diagonal striped columns*), and PANC-1 (*horizontal striped columns*) were incubated for 24 hours with GDNF (0, 10, and 100 ng/mL) in serum-free medium. Microscopic images are representative pictures from three independent experiments of invading pancreatic cancer cells on the lower surface of the membrane. Magnification, ×200. *Columns,* mean number of invading cells per high power field; bars, SD. *, $P < 0.01$ versus control; #, $P < 0.01$ versus Capan-2 and MIA PaCa-2 cells. *C,* the MEK inhibitor PD98059 and PI3K inhibitor wortmannin inhibit the GDNF-induced increase in MIA PaCa-2 invasion. MIA PaCa-2 cells were pretreated with PD98059 or wortmannin followed by GDNF for 24 hours in serum-free medium. *Columns,* mean number of invading MIA PaCa-2 cells per high power field; *bars,* SD. *, $P < 0.01$ versus control; #, $P < 0.01$ versus GDNF 100 ng/mL. *D* and *E,* the GDNF-induced increase in cell invasion is mediated by RET. MIA PaCa-2 cells were preincubated with anti-RET antibodies (*RET ab*) or transfected with RET siRNA followed by GDNF for 24 hours. Isotype-matched control immunoglobulins (*IgG*) and scrambled siRNA served as appropriate controls. *Columns,* mean number of invading MIA PaCa-2 cells per high power field; *bars,* SD. *, $P < 0.01$ versus control; #, $P < 0.01$ versus GDNF 100 ng/mL. *F,* successful down-regulation of RET receptor protein in MIA PaCa-2 cells was confirmed by immunoblotting. Reprobing with an anti-β-actin antibody served as a control (*con.*).

G691S RET polymorphism effect on downstream signaling. Having shown that pancreatic cancer lines with the G691S RET polymorphism respond strongly to GDNF, we investigated whether this RET variant amplifies downstream signaling pathways. GDNF dose-dependently stimulated ERK phosphorylation in AsPC-1, Capan-2, MIA PaCa-2, and PANC-1 cells (Fig. 5*A* and *B*). Fairly robust baseline ERK activation was observed in all cell lines in the absence of GDNF, which can be explained by the known amplified autocrine growth signals in pancreatic cancer cells (4). Nevertheless, whereas GDNF strongly activates ERK in Capan-2 and MIA PaCa-2 cells (G691S RET positive), it had only a weak effect on AsPC-1 and PANC-1 (G691S RET negative) cells. Time-dependent analysis showed that GDNF-induced ERK phosphorylation occurred earlier and lasted longer in G691S RET–positive cells ($P < 0.01$; Fig. 5*C* and *D*). GDNF also stimulated AKT phosphorylation in a dose- and time-dependent manner. However, there was no difference between G691S RET–negative and G691S RET–positive cells (not shown). To unequivocally show that the G691S RET polymorphism is sufficient for modifying downstream signaling pathways, murine fibroblasts lacking RET were transfected with the full-length wild-type RET (WT-RET) or mutated RET (*G691S RET*) gene. Successful transfection and overexpression was confirmed by immunoblotting. Compared to WT-RET, fibroblasts transfected with *G691S RET* showed an 8-fold increase in baseline ERK phosphorylation (Fig. 5*E*).

RET $Tyr^{1,062}$ has been shown to be crucial for activation of the downstream RAS/ERK pathway. Fukuda et al. described that $Tyr^{1,062}$-mediated lamellipodia formation in neuronal cells was inhibited by phosphorylated Tyr^{687}, which blocked $Tyr^{1,062}$-induced Rac GEF activity (34). To show whether the additional serine residue in G691S RET affects phosphorylation of receptor tyrosine residues, RET protein of transfected fibroblasts was immunoprecipitated and phosphotyrosine levels were measured. Western blot analysis showed that overall RET tyrosine phosphorylation was reduced >50% in G691S RET expressing fibroblasts (Fig. 5*E*).

G691S RET polymorphism enhances cell invasiveness. Our results suggested that the G691S RET polymorphism enhances pancreatic cancer cell invasion by augmenting downstream MAPK signaling. To further substantiate this hypothesis, MIA PaCa-2 and PANC-1 were transfected with WT-RET or G691S RET and assessed for their invasiveness in the presence or absence of GDNF. Successful transfection was confirmed by immunoblotting,

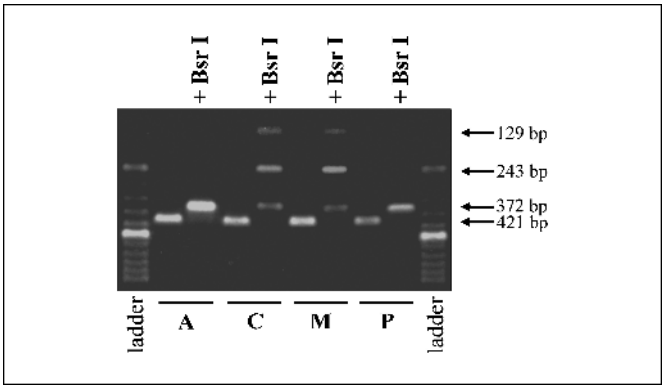

Figure 4. G691S RET receptor polymorphism in pancreatic cancer cells. RET exon 11 was amplified with PCR and digested with *Bsr*I. The wild-type 421 bp RET fragment contains one *Bsr*I site, whereas the G691S RET polymorphism creates an additional restriction site for *Bsr*I (wild type: 49 and 372 bp fragments; G691S RET heterozygous: 49, 129, 243, and 372 bp fragments). After enzymatic digestion of the 421 bp PCR fragment with *Bsr*I, digested and undigested products were separated on an agarose gel. The small 49 bp fragment is not visible on the gel. *A,* AsPC-1; *C,* Capan-2; *M,* MIA PaCa-2; *P,* PANC-1.

showing similar WT-RET and G691S RET protein levels (Fig. 6*A*). Overexpression of G691S RET in both MIA PaCa-2 and PANC-1 cells increased baseline invasion by about 400%, whereas overexpression of WT-RET had no measurable effect ($P < 0.01$; Fig. 6*B*). G691S RET–transfected cells showed a marked increase in ERK phosphorylation (Fig. 6*C*). Transfection of WT-RET in G691S RET–positive MIA PaCa-2 cells partially attenuated the GDNF-induced increase in invasion ($P < 0.05$), whereas it had no additional effect in G691S RET–negative PANC-1 cells (Fig. 6*D*). The reduced effect of GDNF on invasion in WT-RET transfected MIA PaCa-2 cells correlated to a decreased GDNF-induced ERK activation (Fig. 6*E*).

G691S RET polymorphism in tumors. The *in vitro* data indicated that the G691S RET receptor polymorphism may function as a genetic modifier affecting the responsiveness of pancreatic cancer cells to GDNF. We then evaluated randomly selected 52 surgical specimens of patients with histologically verified pancreatic ductal adenocarcinomas. The G691S RET polymorphism was found in 37% (19 of 52) of primary tumors. Seventeen tumors were heterozygous and two were homozygous for the *G691S RET* allele, resulting in an allelic frequency of 20% in primary pancreatic tumors. Histopathologically tumor-free resection margins of the same surgical specimen, i.e. matched normal pancreas, were assessed. The G691S RET polymorphism was found in 31% (16 of 52) of the matched normal pancreas with an allelic frequency of 15% ($P = 0.364$). Interestingly, in 5 of 19 patients (26%) with the G691S RET polymorphism the RET variant seemed to accumulate in the pancreatic tumors (three patients with G691S RET heterozygous tumors but wild-type control tissues and two patients with G691S RET homozygous tumors but G691S RET heterozygous resection margins), indicating a somatic mutation. Immunohistochemical analysis revealed similar expression of the RET protein in pancreatic adenocarcinomas with or without the G691S RET polymorphism (Supplementary Fig. S1).

Discussion

Our study clearly shows that GDNF increases proliferation and invasion of pancreatic cancer cells. The effect of GDNF was

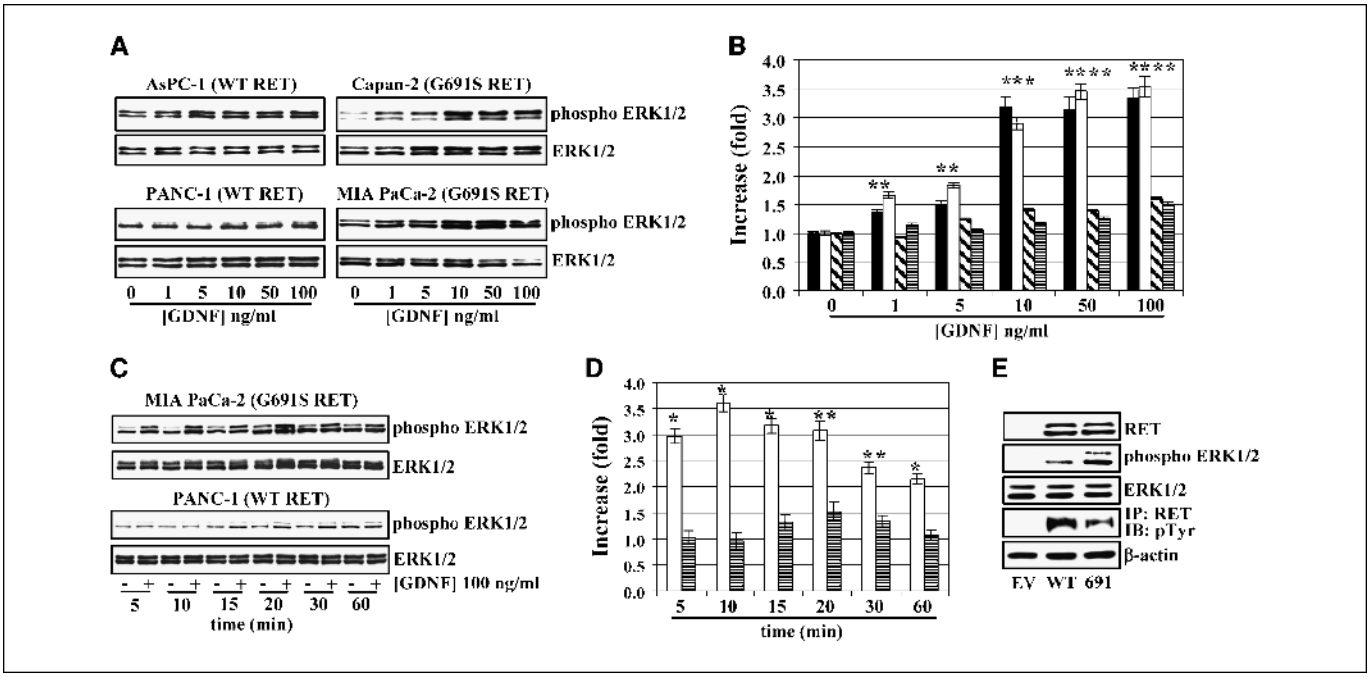

Figure 5. The effect of GDNF on ERK phosphorylation. *A* to *D,* Capan-2 (*black columns*), MIA PaCa-2 (*white columns*), AsPC-1 (*diagonal striped columns*), and PANC-1 (*horizontal striped columns*) were treated with incremental concentrations of GDNF (1, 5, 10, 50, and 100 ng/mL) for 15 minutes in serum-free medium or with 100 ng/mL GDNF for 5, 10, 15, 20, 30, or 60 minutes. Total cell lysates were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with phospho-ERK1/2 antibodies. Reprobing with antibodies detecting total ERK1/2 was used as loading control. Blots are representative images from three independent experiments. *, $P < 0.01$ versus control. *E,* NIH/3T3 fibroblasts were transfected with WT-RET (*WT*) or G691S RET receptor (*691*). Total cell lysates were probed with antibodies against RET, phospho-ERK1/2, total ERK1/2, and β-actin. Cell lysates were immunoprecipitated (*IP*) with RET antibodies and probed with anti-phosphotyrosine antibodies (*pTyr*). *IB,* immunoblotting. Blots are representative images from at least three independent experiments. NIH/3T3 cells transfected with an empty vector (*EV*) served as negative controls.

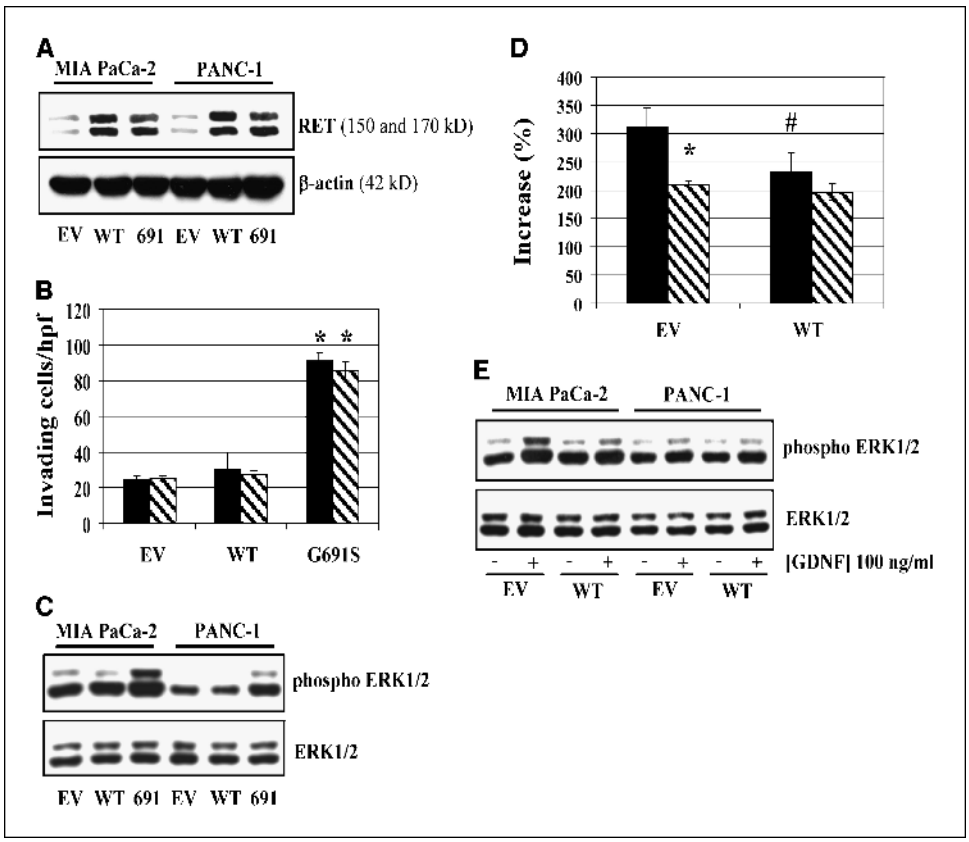

Figure 6. The effect of the G691S RET receptor polymorphism on invasion. *A,* MIA PaCa-2 and PANC-1 cells were transfected with WT-RET or G691S RET receptor. An empty vector served as a negative control. Successful transfection was confirmed by immunoblotting. *B* and *C,* the baseline invasive capacity of transfected MIA PaCa-2 (*black columns*) and PANC-1 (*horizontal striped columns*) cells was determined by Matrigel invasion assays. *Columns,* mean number of invading pancreatic cancer cells per high power field; *bars,* SD. *, $P < 0.01$ versus empty vector. Activation of ERK1/2 in transfected MIA PaCa-2 and PANC-1 cells was determined by immunoblotting. Blots are representative images from at least three independent experiments. *D* and *E,* transfected MIA PaCa-2 (*black columns*) and PANC-1 (*diagonal striped columns*) cells were stimulated with GDNF (100 ng/mL) for 24 hours and tumor cell invasion was determined by Matrigel invasion assays. *Columns,* percentage increase of GDNF-treated cells compared with vehicle-treated cultures; bars, SD. *, $P < 0.01$ versus MIA PaCA-2; #, $P < 0.01$ versus empty vector. Activation of ERK1/2 in transfected MIA PaCa-2 and PANC-1 cells challenged with GDNF was determined by immunoblotting.

completely abrogated by a MEK-1 inhibitor, demonstrating the requirement of the MAPK pathway in these processes and corroborating recent findings (35). We found that the growth-stimulating and proinvasive properties of GDNF differed strongly between various pancreatic cancer cell lines, which could not be explained by differences in the expression of GDNF receptors. The most salient finding in our study was that the effect of GDNF on proliferation and invasion was more prominent in pancreatic cancer cells that harbor the G691S RET receptor polymorphism. In this report, we presented data from two pancreatic cancer cell lines with (Capan-2 and MIA PaCa-2) and without (AsPC-1 and PANC-1) the G691S RET polymorphism. Similar data were obtained with BxPC-3 (WT-RET) and HPAF-II (G691S RET–positive) cells.

The allelic frequency of the G691S RET polymorphism in the normal population has been reported to be 15% to 20% (33, 36, 37). The G691S RET variant seems to be more frequently expressed in sporadic medullary thyroid carcinomas and in radiation-induced thyroid tumors (36, 38). In contrast, the G691S RET polymorphism is underrepresented in patients with Hirschsprung disease (39). The frequency of the *G691S RET* allele in MEN 2 patients does not significantly differ from healthy controls, but it is associated with an earlier onset of the disease (20, 40). From these studies, it seems that the G691S RET polymorphism may function as a low-penetrance gene or genetic modifier of certain thyroid tumors. However, no study has investigated the cellular and molecular mechanisms by which the G691S RET receptor polymorphism may alter the cellular phenotype. Ligand-independent constitutive receptor dimerization and altered substrate specificity leading to enhanced constitutive kinase activity are proposed functional results of oncogenic RET receptor mutations (13, 41).

Pancreatic cancer cell lines with the G691S RET receptor polymorphism showed significantly more robust GDNF-induced ERK activation. Fibroblasts transfected with G691S RET showed enhanced ligand-independent ERK phosphorylation. This was associated with reduced phosphorylation of RET tyrosine residues. GDNF triggers transphosphorylation on multiple tyrosine residues. $Tyr^{1,062}$-mediated lamellipodia formation in neuronal cells was inhibited by phosphorylated Tyr^{687} (34). The finding that protein kinase A–mediated phosphorylation of Ser^{696} inhibited Tyr^{687} phosphorylation, thereby promoting lammelipodia formation, indicated a regulatory role of serine residues in RET signaling (34). Based on our findings and the structural proximity to Ser^{696}, we hypothesize that the G691S RET polymorphism creates an additional serine phosphorylation site. Increased serine phosphorylation at this site may further inhibit Tyr^{687} phosphorylation and, hence, activate downstream RET signaling.

Overexpression of G691S RET, but not WT-RET, profoundly increased invasion of pancreatic cancer cells in the absence of GDNF, indicating that the G691S RET polymorphism indeed modulates the cellular phenotype. Stimulation of G691S RET receptor–transfected pancreatic cancer cells with GDNF further enhanced invasive capacity by only 20% (not shown), presumably because intracellular signaling pathways were already almost maximally activated by the ectopically overexpressed G691S RET receptor.

Our findings are similar to a recent report that showed ligand-independent activation of downstream signaling molecules in RET-familial medullary thyroid carcinoma mutants Y791F and S891A

(42). However, although ectopically overexpressed RET mutants may suggest ligand independency, cells that express RET receptor variants at endogenous levels may still be responsive to GDNF. This is supported by our study in which GDNF had a robust effect on pancreatic cancer cells with endogenous levels of G691S RET, but only a weak effect in G691S RET overexpressing cell lines. Overexpression of WT-RET in G691S RET receptor harboring pancreatic cancer cells significantly reduced GDNF-induced invasion, supporting our hypothesis that the G691S RET receptor polymorphism is associated with a biologically more aggressive phenotype. Taken together, our *in vitro* findings suggest that the G691S RET receptor polymorphism enhances the sensitivity of pancreatic cancer cells to GDNF by elevating baseline MAPK activation.

We also found the G691S RET polymorphism in human pancreatic ductal adenocarcinomas. Using immunohistochemistry, no obvious difference in the expression levels of RET between tumors with and without the G691S RET polymorphism were observed. Similar to a previous report in MEN 2 patients (20), the frequency of the *G691S RET* allele did not differ significantly between primary pancreatic tumors and matched control tissues. However, the observation that in 5 of 19 patients (26%) with the G691S RET polymorphism the RET variant seems to accumulate in the primary pancreatic tumors (three patients with G691S $RET^{+/-}$ tumors but G691S $RET^{-/-}$ control tissues and two patients with G691S $RET^{+/+}$ tumors and G691S $RET^{+/-}$ resection margins) indicates a somatic event and suggests a role of the G691S RET polymorphism as a low-penetrance gene. Unfortunately, the retrospective analysis of archived pancreatic cancer samples limited our efforts to detect any correlation between the G691S RET polymorphism and clinical variables. Moreover, we did our analyses on a selected cohort of patients with low-stage, resectable tumors, which comprise only a small fraction of patients with pancreatic cancers. It is conceivable albeit speculative that in the entire patient population with pancreatic cancers the G691S RET polymorphism is more frequently expressed in the primary tumors than our data suggest. If the G691S RET polymorphism—as our *in vitro* data indicate—correlates to a more aggressive phenotype, the majority of these patients may not be candidates for surgical resection and, therefore, may elude our analysis. Nevertheless, prospective studies with the aim to correlate the presence of the G691S RET polymorphism with pancreatic cancer growth, local tumor recurrence and overall patient survival are warranted.

In summary, we found that GDNF stimulates proliferation and invasion of pancreatic cancer cells by RET receptor–mediated activation of the ERK MAPK pathway, further highlighting a critical role of GDNF in pancreatic cancer progression. The G691S RET polymorphism was also detectable in human pancreatic cancers and in some cases seemed to be a somatic event. This finding possibly identifies a subset of patients with biologically more aggressive tumors.

Acknowledgments

Received 8/10/2005; revised 9/26/2005; accepted 10/11/2005.

Grant support: Hirshberg Foundation for Pancreatic Cancer Research (H.A. Reber), Martin H. Weil Pancreatic Cancer Research Fund (D.S.B. Hoon), and NIH grant CA104027 (G. Eibl).

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