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Genetic Alterations of the Transforming Growth Factor β Receptor Genes in Pancreatic and Biliary Adenocarcinomas[1]

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Abstract

Transforming growth factor β (TGF-β) is an extracellular ligand that binds to a heterodimeric receptor, initiating signals that regulate growth, differentiation, and apoptosis. Many cancers, including pancreatic cancer, harbor defects in TGF-β signaling and are resistant to TGF-β-mediated growth suppression. Genetic alterations of *DPC4*, which encodes a DNA binding protein that is a downstream component of the pathway, most frequently occur in pancreatic and biliary carcinomas. We searched for other targets of mutation of the TGF-β pathway in these cancers. We report somatic alterations of the TGF-β type I receptor gene *ALK-5*. Homozygous deletions of *ALK-5* were identified in 1 of 97 pancreatic and 1 of 12 biliary adenocarcinomas. A germ-line variant of *ALK-5*, presumably a polymorphism, was identified, but no somatic intragenic mutations were identified upon sequencing of all coding regions of *ALK-5*. Somatic alterations of the TGF-β type II receptor gene (*TGFBR2*) were identified in 4 of 97 (4.1%) pancreas cancers, including a homozygous deletion in a replication error-negative cancer and three homozygous frameshift mutations of the poly(A) tract of the TGF-β type II receptor in replication error-positive cancers. We also studied other related type I receptors of the TGF-β superfamily. In a panel of pancreas cancers preselected for loss of heterozygosity at the *ALK-1* locus, sequencing of all coding exons of the *ALK-1* gene revealed no alterations. No homozygous deletions were detected in the *ALK-1*, *ALK-2*, *ALK-3*, or *ALK-6* genes in a panel of 86 pancreatic cancer xenografts and 11 pancreatic cancer and 22 breast cancer cell lines. The rate of genetic inactivation of TGF-β pathway members was determined in 45 pancreatic cancers. Eighty-two % of these pancreatic cancers had genetic inactivation of the *DPC4*, *p15*, *ALK-5*, or *TGFBR2* genes. Our results indicate that the TGF-β type I and type II receptor genes are selective targets of genetic inactivation in pancreatic and biliary cancers.

Introduction

The TGF-β[3] type I receptor ALK-5 forms a heterodimer with the TGF-β type II receptor to mediate signaling of TGF-β ligands. Downstream components of the pathway include *SMAD2* (*JV18-1*, *MADR2*), *SMAD3*, and *SMAD4* (*DPC4*, *MADH4*). Signaling initiated after binding of TGF-β and related ligands to their cognate receptors leads to heteromerization and nuclear translocation of the Smad proteins and the transcriptional activation of target genes (reviewed in Ref. 1).

Although *SMAD4*, *SMAD2*, and *TGFBR2* are tumor suppressor genes that are known to be genetically inactivated in human cancer (2–7), the genetic inactivation of *ALK-5* has not been identified (8).

Kim *et al.* (9) reported a prostate cancer cell line with Southern blot abnormalities of the *ALK-5* gene. Reduced or absent expression of *ALK-5* has been demonstrated multiple tumor types (10–13). Interstitial deletions near the *ALK-5* locus on chromosome 9q21 have been reported in acute myelocytic leukemia (reviewed in Ref. 10), but *ALK-5* has not been confirmed as the target of these deletions (8). Sequence analysis of *ALK-5* in acute myelocytic leukemia did not identify it as a target of somatic mutation (8). Although TGF-β resistance is a common phenomenon in many cancer types, the tumor suppressor genes of the TGF-β/DPC4 pathway are not reported to be common targets of genetic alteration (6, 8, 14).

Because pancreatic and biliary cancers have an exceptionally high rate of genetic inactivation of one member of this pathway, *SMAD4*, we searched for genetic targets of mutation within the TGF-β pathway by studying the TGF-β receptors and other related members of the TGF-β receptor family in these cancers.

Materials and Methods

Patient Population and Tissue Samples. Normal and tumor specimens were obtained from pancreatic ductal adenocarcinomas resected at The Johns Hopkins Hospital (Baltimore, MD) by pancreaticoduodenectomy (Whipple procedure). The institutional review committee on clinical investigation reviewed and approved the collection of the tissue samples for genetic analysis. Pancreatic carcinoma xenografts were established from the primary cancers as described previously, and carcinoma and normal tissues were stored at −70°C (15). Genomic DNA was prepared from 11 pancreatic adenocarcinoma cell lines. The pancreatic cancer cell lines were: AsPc-1, BxPc3, Capan1, Capan2, Panc1, Su86.86, CFPAC1, MiaPaca2, and Hs766T (American Type Culture Collection, Manassas, VA); Colo357 (ECACC, Salisbury, United Kingdom); and PL45, a low-passage cell line established in our laboratory.

Homozygous Deletion Determination. A panel of pancreatic and biliary adenocarcinomas xenografts, as well as pancreatic cancer cell lines, were screened for homozygous deletions by PCR. Because mouse stroma was present in xenografts, in instances in which the genomic structure of a given gene was unknown, DNA amplification was performed on genomic templates using exonic primers that were proven not to amplify mouse genomic DNA. All homozygous deletions were confirmed using a minimum of three independent PCRs. Independent PCRs using nearby primer sets were also tested to rule out primer site polymorphism. Homozygous deletions were then confirmed using duplex PCR, as described previously (2). The *ALK-5* and *TGFBR2* homozygous deletions were also confirmed using Southern blot analysis, as described previously (2).

RT-PCR. RT-PCR was performed from total RNA or mRNA (Micro QuickPrep; Pharmacia) isolated from xenografts of pancreatic adenocarcinoma. cDNA was amplified for 45 cycles using appropriate primers. RT-PCR was performed to determine gene expression and as an intermediate step in the mutation analysis of the *ALK-6* gene. RT-PCR was also performed to create templates for use in the *in vitro* synthesized protein truncation assay for exon 4 of the *TGFBR2* gene, as described previously (16).

LOH Analysis. LOH at the *ALK-5* region was determined using the microsatellite markers *D9S272*, *D9S154*, *D9S258*, and *D9S1782*, as described (16). For most cancers, the corresponding normal DNA was not available for comparison. In such cases, LOH was presumed present if one allele was present in all of the polymorphic microsatellite markers tested. LOH at the

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[3] The abbreviations used are: TGF-β, transforming growth factor β; ALK, activin receptor-like kinase; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity; RER^+ and RER^-, replication error-positive and -negative, respectively.

TGFBR2 locus was performed using microsatellite markers *D3S1567*, *D3S1609*, and *D3S3547*. LOH analysis at the *ALK-1* locus was determined using the markers *D12S368*, *D12S325*, *D12S1707*, and *D12S312*. LOH at the *ALK-6* locus was determined with markers *D6S975* and *D6S1656*.

Sequencing. Prior to sequencing, PCRs were incubated with exonuclease I and shrimp alkaline phosphatase (Amersham), according to the manufacturer's recommendations. Sequencing of PCR products was performed in microtiter plates, as recommended by the manufacturer (Sequitherm Excel; Epicentre Technologies, Madison, WI). All mutations were confirmed by the sequencing of independent PCR products.

Dot Hybridization. The frequency of the R168C polymorphism (a C-to-T alteration at nucleotide 578), was determined by dot hybridization, as described previously (17). A total of 246 patients with pancreatic adenocarcinoma and 91 patients with miscellaneous carcinomas were screened. The region of the *ALK-5* gene containing the polymorphism was amplified by PCR. Each PCR product was immobilized on a nylon membrane with 0.5 M NaOH and probed with a ^{32}P end-labeled 18-mer that was complementary to the C-to-T alteration at position 578. The polymorphism was positioned to the center of the oligomer to minimize nonspecific annealing of the mutant primer to the wild-type sequence. The membrane included a PCR product known to contain the R168C polymorphism. Following hybridization, the membrane was serially washed in high salt buffer at serial temperatures and autoradiographed.

Clinicopathological Review. The clinical histories of all patient whose carcinoma contained an *ALK-5* or an *TGFBR2* genetic abnormality and the histopathologies of the resected carcinomas were reviewed.

Results

***ALK-5* Homozygous Deletion Screening.** A homozygous deletion of *ALK-5* was identified in 1 of 86 pancreatic cancers (PX321), 0 of 11 pancreatic cancer cell lines, and 1 of 12 biliary cancers (PX109). The homozygous deletions were confirmed using multiple PCR primer sets spanning the coding sequence of *ALK-5* (primers available on our Web site (http://www.path.jhu.edu/pancreas). Duplex PCR confirmed the homozygous deletions (Fig. 1). All nine exons of *ALK-5* were lost in PX321, and in PX109, exon 1 of *ALK-5* was retained whereas exons 2–9 were lost (Table 1). Whereas most pancreatic cancers demonstrated variable levels of expression of *ALK-5* message by RT-PCR, no expression of *ALK-5* was detected by RT-PCR in the two carcinomas with homozygous deletions (data not shown). Further confirmation of the homozygous deletion was obtained by Southern blot analysis. An *ALK-5* PCR product (249 bp) was used to probe *Bgl*II-digested 10-μg samples of genomic DNA transferred onto a nylon membrane. The *ALK-5* PCR probe contained a *Bgl*II restriction site, creating two bands in four control lanes (genomic DNA from three pancreas cancers and one normal duodenum) on Southern blot autoradiographs; these bands were absent in the PX321 and PX109 DNA (data not shown). *ALK-5* has been located to 364cR on a radiation hybrid map (marker WI-7314). We tested two additional markers localized to 364 cR on the chromosome 9 radiation hybrid map (http://www.wi.mit.edu; Whitehead Institute). Both markers were retained in PX321, and one marker (WI-5802) was deleted in PX109. A parallel xenograft of one of the cancers (PX321, established from an independent sample from the primary tumor) was available for analysis and also demonstrated homozygous deletion of *ALK-5*. DNA template quality was confirmed in the cases with homozygous deletions by successful amplification of a large panel of genome-wide microsatellite markers (>100 markers). Information regarding the frequency of homozygous deletion in pancreatic cancer is provided by a study of allelic loss on chromosome 1p.[4] In that study, 43 pancreatic cancer xenografts were analyzed using 44 randomly distributed chromosomal microsatellite markers. No homozygous deletions of any marker were identified in PX321, PX17 (see below), or any other pancreatic cancer xenografts. In addition, concurrent probing of 25 Southern blots of pancreatic cancer xenografts with >40 Image Consortium clones failed to identify any homozygous deletions.[4] Similarly, we performed homozygous deletion screening of other genes of the TGF-β type I receptor superfamily in 86 pancreatic adenocarcinoma xenografts and 11 pancreatic carcinoma cell lines. We were unable to identify any homozygous deletions of the *ALK-1*, *ALK-2*, *ALK-3*, and *ALK-6* genes (see below). Similarly, of the studies that have evaluated candidate tumor suppressor genes in human cancers by screening for homozygous deletion, most have failed to identify any cancers with homozygous deletion (http://www.NOGO.org). These data suggest that it is rare to observe homozygous deletion as a product of background genetic instability or chance alone.

Table 1 *Homozygously deleted markers at the ALK-5 locus in pancreatic and biliary cancer*

Marker	PX109 (biliary carcinoma)	PX321 (pancreatic carcinoma)
ALK-5 exon 1	Retained	Lost
ALK-5 exon 2	Lost	Lost
ALK-5 exon 3	Lost	Lost
ALK-5 exon 4	Lost	Lost
ALK-5 exon 5	Lost	Lost
ALK-5 exon 6	Lost	Lost
ALK-5 exon 7	Lost	Lost
ALK-5 exon 8	Lost	Lost
ALK-5 exon 9	Lost	Lost
WI-5802	Lost	Retained
WI-10515	Retained	Retained

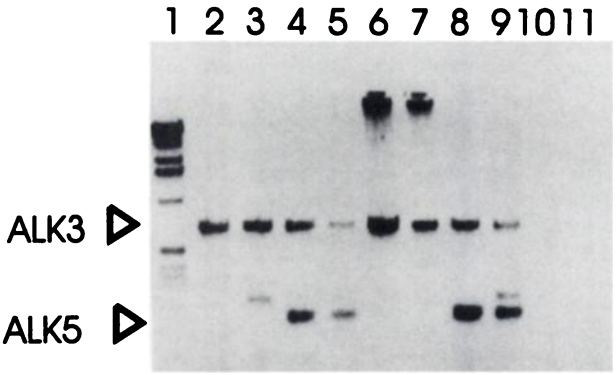

Fig. 1. Duplex PCR using primers from the *ALK-5* gene exon 9 (PCR product, 249 bp) and a control locus in the *ALK-3* gene (PCR product, 742 bp). *Lane 1*, molecular weight marker; *Lanes 2* and *3*, PX321 (pancreatic adenocarcinoma xenograft); *Lanes 4* and *5*, PX202 (biliary adenocarcinoma xenograft); *Lanes 6* and *7*, PX109 (biliary adenocarcinoma xenograft); *Lanes 8* and *9*, PX264 (biliary adenocarcinoma xenograft); *Lanes 10* and *11*, no template. *Lanes 2* and *3*, *6*, and *7* demonstrate absence of the *ALK-5* PCR product. PCR products from the nondeleted reference gene, *ALK-3*, were retained in both carcinomas containing the *ALK-5* homozygous deletions.

***ALK-5* Mutational Analysis.** The genomic structure of *ALK-5* was determined by a combination of direct bacterial artificial chromosome sequencing and long PCR (the genomic structure of *ALK-5* has since been submitted to GenBank by V. F. Vellucci and M. Reiss, accession no. AF054590). Sequence analysis of pancreatic cancer xenografts preselected for LOH on chromosome 9q22 identified one nucleotide variant (C to T, R168C) of *ALK-5*. This polymorphism was present in the constitutional DNA from this patient. It was not present in the genome of 246 patients with pancreatic cancer or in 91 patients with miscellaneous cancers. *ALK-5* codon 168 is located in the intracellular portion of *ALK-5*, 17 amino acids upstream of the GS domain and 3 amino acids from Ser-165. Mutation of the serines and threonines in the GS domain and of Ser-165 interferes with type I receptor function

[4] W. H. Hilgers and S. E. Kern, High-resolution deletion mapping of chromosome arm 1p in pancreatic cancer identifies a major consensus region at 1p35, submitted for publication.

(18). The carrier of the R168C polymorphism developed her cancer at age 72 and did not have any additional personal or family history of cancer. No other sequence variants were identified (8).

Clinicopathological Features. The pancreatic cancer xenograft (PX321) was derived from the primary tumor of a 57-year-old female who underwent a Whipple resection for a moderately differentiated 5-cm carcinoma that did not have lymph node metastases. The patient is alive 2 years after her operation. The biliary carcinoma xenograft (PX109) was prepared from a 68-year-old man who underwent a Whipple resection for a 2-cm moderately differentiated carcinoma without lymph node metastasis.

***TGFBR2* Mutational Analysis.** The *TGFBR2* gene was screened for homozygous deletions. A single homozygous deletion was detected in a panel of 86 pancreatic cancer xenografts and in none of 22 breast cancer cell lines. The deletion of PX17 included all exons of the *TGFBR2* gene. The homozygous deletion of *TGFBR2* has been reported previously in two gastric carcinomas (7). Presumptive LOH at chromosome 3p21 was demonstrated using *D3S1567*, *D3S1609*, and *D3S3547* in 24 of 81 of pancreatic cancer xenografts, 2 of 11 pancreatic cancer cell lines, 2 of 2 breast cancer xenografts, and 7 of 16 breast cancer cell lines. The 35 carcinomas with 3p21 LOH were sequenced for mutations by cycle sequencing of exons 2, 3, 5, 6, and 7. Exon 4 was screened using the *in vitro* synthesized protein truncation assay. Expression of the TGF-β type II receptor was detected by RT-PCR in cases with LOH at chromosome 3p21. Three pancreatic cancer xenografts were found to have frameshift mutations in the poly(A)$_{10}$ tract within exon 3 of the *TGFBR2* gene (Fig. 2).

Pathological Differences between Pancreatic Cancers with RER$^+$ and RER$^-$ *TGFBR2* Mutations. The clinical and pathological characteristics of the RER$^+$ carcinomas have been described elsewhere (19). The tumors with TGFBR2 mutations had a medullary histological appearance and wild-type K-*ras* sequences that were characteristic of RER$^+$ pancreatic cancers (19). The RER$^-$ pancreatic carcinoma with the homozygous deletion of *TGFBR2* did not display any unique pathological or clinical features. No other mutations of the *TGFBR2* gene were identified.

***ALK-1* Mutational Analysis.** The *ALK-1* gene has been localized to chromosome 12q (20). Four of 11 pancreatic carcinoma cell lines and 16 of 81 pancreatic carcinoma xenografts had LOH of this region and were selected for mutational analysis. In addition, we studied a patient with hereditary hemorrhagic telangiectasia (a cause of which includes germ-line mutations of *ALK-1*) who had developed pancreatic cancer. No mutations were detected in the coding region of *ALK-1*.

***ALK-6* Mutational Analysis.** The *ALK-6* gene was localized to chromosome 6 using a yeast artificial chromosome panel (YAC 863C11) and confirmed using monochromosomal somatic cell hybrids (Coriel). Sixteen of 81 pancreatic carcinoma xenografts and 7 of 11 pancreatic carcinoma cell lines had LOH at markers *D6S975* and *D6S1656*. In 15 of 23 pancreatic carcinomas, RT-PCR products were screened for mutations in the 3′ portion of *ALK-6* gene by cycle sequencing (from nucleotide 1332 to the stop codon at 1782, GenBank accession no. U89326). No mutations were detected in *ALK-6*.

Fig. 2. Mutations of the poly(A) tract of the *TGFBR2* gene in pancreatic cancer. *Lanes 1–3*, sequences of three RER$^+$ pancreatic carcinoma xenografts with mutations the poly(A)$_{10}$ tract within exon 3 (PX26, PX196, and PX287, respectively); *Lane 4*, constitutional DNA. The "A" lane sequence is shown. *0*, *+1*, and *−1* correspond to control, insertion, and deletion mutants of the poly(A) tract, respectively.

Table 2 *Genetic inactivation of the TGF-β pathway-related genes in pancreatic cancer*

Gene	Selected series (%)[a]	Reference series %[b]	Reference
TGFBR2	4/45 (9)	4	This study
ALK-5	1/45 (2)	1	This study
DPC4	26/45 (58)	50	Hahn *et al.* (2)
p15	18/45 (40)	30	Rozenblum *et al.* (21)

[a] Totals are presented only for the selected series to assure complete ascertainment. Pancreatic carcinomas were selected for inclusion in this analysis if the mutation status of *DPC4*, *ALK-5*, *TGFBR2*, and *p15* was known.

[b] Rates of inactivation for *DPC4*, *p15*, *TGFBR2*, and *ALK-5* genes were ascertained without prior selection. Thirty-five of 45 cancers had genetic inactivation of one gene in the pathway, and 10 of 45 had two genes. In 9 cancers, both *DPC4* and *p15* were inactivated, and in one cancer, both *DPC4* and *ALK-5* were inactivated.

***DPC4* and *p16* Genes.** Studies of genetic alterations in the *DPC4* and *p16* genes revealed that the *ALK-5* homozygous deletions were accompanied by homozygous deletion of *p16* in both the pancreatic and the biliary adenocarcinoma and by homozygous deletion of *DPC4* in one case (biliary carcinoma). *DPC4* was sequenced in the remaining carcinomas that harbored *ALK-5* or *TGFBR2* mutations. No sequence alterations of *DPC4* were identified.

TGF-β Pathway Inactivation in Pancreatic Cancer. Table 2 summarizes our mutational analysis of a set of 45 pancreatic cancers. Each of the 45 cancers has been studied for genetic alteration of the mutational targets related to the TGF-β pathways including the TGF-β receptors and *DPC4* and *p15* genes. Most of the results of the *DPC4* and *p15* genes have been presented separately elsewhere (2, 21). Over 80% of pancreatic cancers had genetic inactivation of a member related to these pathways.

Discussion

This is the first report of the genetic inactivation of the TGF-β type I receptor, *ALK-5*, in human cancer. The finding that *ALK-5* is a mutationally targeted tumor-suppressor gene adds to the substantial body of evidence regarding the tumor-suppressive function of the TGF-β pathway (1–14). Many cancer cell lines fail to respond to the TGF-β ligand by growth inhibition, consistent with an abrogation of this pathway during tumorigenesis (22). We did not identify somatic sequence alterations of *ALK-5*. Indeed, homozygous deletions predominate for some tumor suppressor genes such as *p16* (15). As a practical concern, the genetic inactivation of *ALK-5* by homozygous deletion would be expected to make identification difficult in studies of unenriched primary tumors. Although perhaps unlikely, the deletion of *ALK-5* does not exclude the possibility of a neighboring gene as a target of selection.

Clonal selection theory would predict that, within a neoplastic clone, the inactivation of one gene in a given tumor-suppressive pathway affords a selective growth advantage that would abrogate the need to inactivate a second gene in the same pathway (23). The mutual exclusiveness of *p53/MDM2* and of *p16/RB1/CDK4* genetic alterations has provided support for the functional studies that have placed each of these gene sets, respectively, in the same tumor-suppressive pathway. The corollary of this property is that, when mutations of several genes are found in a single cancer, then these genes are most likely parts of functionally distinct pathways. The coexistent homozygous deletion of both the *ALK-5* and the *p16* genes in one pancreatic cancer and the homozygous deletion of the *TGFBR2* gene and *p16*

methylation in another case imply that the TGF-β and the p16/Rb pathways mediate functionally distinct tumor-suppressive functions (23). Similarly, the coexistent homozygous deletion of both *ALK-5* and *DPC4* in biliary cancer implies that these genes mediate some nonoverlapping functions.

DPC4 inactivation should inactivate certain non-TGF-β pathways (1, 24). We therefore extended our study to include the ALKs that serve as receptors for other ligands of the TGF-β superfamily, such as activin and bone morphogenic proteins. ALK-1 binds to TGF-β and activin type II receptors (20). ALK-2 is a receptor for activin and BMP7, whereas ALK-6 (BMP-1B) is a receptor for bone morphogenic proteins (BMP-4 and BMP-7; Refs. 25 and 26). Both of these genes function in growth/differentiation pathways but were not identified as tumor suppressor genes from our studies.

The TGF-β pathway is affected in most pancreatic cancers (2, 9, 24). Genetic alterations occur at *DPC4* (~50%), *TGFBR2* (~4%), and *ALK-5* (~1%). In addition, p15 can participate in TGF-β-mediated cell cycle arrest (27). p16 is a target of mutations and deletion in neoplasia; these events often also affect the $p14^{ARF}$ and (in the case of homozygous deletion) the $p15^{INK4B}$ genes. Although the selective pressures attributable to loss of $p14^{ARF}$ and p15 in tumors are difficult to specify, the loss of p15 would have the effect of impairing a putative TGF-β effector arm (21, 28). If the genetic inactivation of *p15* (in 30% of pancreatic cancers) is included (21), the cumulative rate of TGF-β pathway defects (due to the genetic inactivation of *ALK-5*, *TGFBR2*, *DPC4*, and *p15*) rises to >80% of pancreatic cancers (Table 2). Other components of this pathway, such as SMAD1-SMAD3 and SMAD5 and SMAD6, have not been demonstrated as targets of inactivation in pancreatic cancer (29). Currently, therefore, *p15* is the only known candidate gene downstream of the TGF-β receptors other than *DPC4* that is commonly involved by genetic inactivation.

Given the high rate of inactivation of the TGF-β pathway in pancreatic and other cancers as shown by functional studies, it is also likely that additional genetic targets within the TGF-β pathway remain to be identified.

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