The Biology Behind

SMAD4/DPC4 and Pancreatic Cancer Survival

Commentary re: M. Tascilar *et al.*, The SMAD4 Protein and Prognosis of Pancreatic Ductal Adenocarcinoma. Clin. Cancer Res., *7:* 4115–4121, 2001.

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Pancreatic cancer is the fourth leading cause of cancer deaths in both men and women in the United States, accounting for ~30,000 deaths annually. It is a deadly disease with a 5-year survival of only 3–5% (1). The diagnosis of pancreatic adenocarcinoma is devastating to patients and their families, because the diagnosis is usually made at a late stage of disease that is not amenable to cancer curative surgery, and is little affected by chemotherapy, radiation therapy, or immunotherapy. Patients have a median survival of only 4–8 months after diagnosis (1). A small number of pancreatic cancer patients are diagnosed when the disease is limited to the pancreas and periampullary region. Surgery (pancreaticoduodenectomy) provides the only potentially curative intervention for the disease with a 5-year survival of 15–20% (1).

SMAD4, also termed *DPC4*,[2] was originally isolated from human chromosome 18q21.1 as a tumor suppressor gene for pancreatic cancer (2). Approximately 55% of pancreatic cancers bear deletions or mutations in *SMAD4/DPC4* (~30% homozygous deletion and ~22%-25% mutation with LOH; Ref. 2). In this issue, Tascilar *et al.* (3) report that among patients undergoing surgical resection of their pancreatic adenocarcinoma, survival of patients whose tumors expressed SMAD4 protein was significantly longer (unadjusted median survival, 19.2 months) as compared with 14.7 months without SMAD4 protein expression ($P = 0.03$; Ref. 3). This SMAD4 survival benefit persisted after adjustment for prognostic factors including tumor size, margin status, lymph node status, pathological stage, blood loss, and use of adjuvant chemoradiotherapy (3). For a disease with such a poor prognosis, the median survival advantage of ~5 months with SMAD4 expression is clinically significant.

Pancreatic cancer is primarily a genetic disease. Inactivation of several tumor suppressor genes, such as *p16, SMAD4,* and *p53,* coupled with activation of the K-*ras* oncogene, are common events in pancreatic cancer (4). Mutation frequencies for *p16, p53,* and K-*ras* are approximately 80, 70, and 90%, respectively (4). Several molecular markers have been investigated previously for their prognostic significance, including *p53* status (5), K-*ras* mutation (5), expression of *bcl-2* (6), *bax* (6), and *TGF-β1* (7). Only the DNA index has been shown consistently to provide prognostic information independent of standard pathological prognostic indicators (8).

SMAD4 is a member of the SMAD family and plays a pivotal role in mediating members of the TGF-β superfamily signal transduction and gene regulation events (9, 10). The major members of the TGF-β family include TGF-βs, activins, and BMPs (9, 10). The TGF-β family members exert a wide variety of biological activities. For example, TGF-β can regulate the proliferation, differentiation, motility, and death of cells. TGF-β can also enhance extracellular matrix formation, promote angiogenesis, and inhibit immune function. Because of their multifunctional nature, TGF-β and related factors can elicit different effects in a cell context-dependent manner (9, 10).

TGF-β signals through two types of transmembrane serine/threonine kinase receptors (9, 10). It binds and brings together the type I and type II receptors (TGFBR1 and TGFBR2). In the resulting complex, the constitutively active TGF-β type II receptor phosphorylates the type I receptor, which then plays a major role in transducing the signal to downstream components to affect gene expression. Other members of the TGF-β family also signal through two types of transmembrane serine/threonine kinase receptors (9, 10). The activin type IB receptor (ACVR1B) and the TGF-β type I receptor (TGFBR1) share >90% homology in their kinase domains, which may explain the finding that TGF-β and activin share some common biological activities, including growth-inhibitory effects.

SMAD proteins can transduce the TGF-β signal from the cell surface to the nucleus (9, 10). On the basis of structural and functional characteristics, the nine members of the SMAD family can be divided into distinct groups. One group includes those pathway-specific SMADs that are phosphorylated by receptor kinases (also called receptor-regulated SMADs). For example, SMAD1 and its close homologues SMAD5 and SMAD8 mediate BMP responses and can be directly phosphorylated by BMP receptor kinases (9, 10). SMAD2 and SMAD3 mediate TGF-β and activin responses and can be directly phosphorylated by the highly homologous TGF-β and activin receptor kinases (9, 10). Phosphorylated receptor-regulated SMADs then form heteromeric complexes with the common partner SMAD4 (co-SMAD; Ref. 11). These heteromeric complexes then move to the nucleus, where SMAD4 contributes to DNA binding and is critical for transcriptional activation (12). SMAD4 is the only member of the SMAD family that can participate in TGF-β, activin, and

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[2] The abbreviations used are: DPC4, deleted in pancreatic carcinoma, locus 4; LOH, loss of heterozygosity; TGF-β, transforming growth factor β; BMP, bone morphorgenetic protein; CDK, cyclin-dependent kinase; APC, adenomatous polyposis coli; VEGF, vascular endothelial growth factor; TSP, thrombospondin.

BMP signaling pathways, which underscores its central role in TGF-β family signaling (9–12).

The most prominent biological activity of TGF-β is its potent inhibition of cell growth in a wide variety of cell types (9, 10). TGF-β inhibits cell proliferation by causing cell cycle arrest at the G_1 phase through regulation of cell cycle components (9, 10). It has been shown that SMAD proteins can mediate the TGF-β growth-inhibitory responses. For example, SMAD2, SMAD3, and SMAD4 together can down-regulate the expression of the c-*myc* proto-oncogene and up-regulate the expression of CDK inhibitors p15 and p21. The induced p15 binds to CDK4 and CDK6 and prevents their interaction with cyclin D. As a result, the CDK inhibitor p27, which is bound to the cyclin D-CDK4, is displaced and binds to the cyclin E-CDK2 complex to inhibit its activity. Thus, the coordinated inhibition of CDK4/6 and CDK2 activities by p15 and the p21 family leads to cell cycle arrest induced by TGF-β (9, 10).

Many cancers, including pancreatic cancer, harbor defects in TGF-β signaling and are resistant to TGF-β-mediated growth suppression (9, 10). TGF-β type II receptor has been shown to be mutated in colon cancer (30%), gastric cancer (15%), endometrial cancer, and a number of others including pancreatic cancer (9, 10). The TGF-β type I receptor is mutated in breast cancer (16%) and several other types of cancers including pancreatic cancer (9, 10). TGF-β receptor genes are usually mutated at low frequency compared with the high frequency of expression defects that affect the receptors (13, 14). The roles of activins and BMPs in human tumorigenesis are just beginning to be unraveled. A recent study found that the activin type IB receptor gene (*ACVR1B*) is also mutated in pancreatic cancer (14). This may not be unexpected, considering that activin can also elicit growth-inhibitory effects, and the ACVR1B also uses the same set of SMAD proteins (SMAD2, SMAD3, and SMAD4) to signal. No mutations have been detected thus far for several other receptors examined, including ALK1 (a type I receptor for both TGF-β and activin), ALK2 (a type I receptor for activin and BMP), ALK3 (a type IA receptor for BMP), and ALK6 (a type IB receptor for BMP) (Ref. 15).

In addition to pancreatic cancer, SMAD4 is also mutated in colon and biliary cancers (30%), and to a lesser extent, in a number of cancers from other tissues, including lung, breast, prostate, ovarian, head and neck, esophageal, gastric, bladder, liver, and kidney (9, 10). SMAD4 is also mutated in a subgroup of patients with familial juvenile polyposis, an autosomal dominant disorder characterized by a predisposition to hamartomatous polyps and cancers of the gastrointestinal tract (9, 10). Several studies have examined the role of SMAD4 in tumorigenesis and in embryonic development using mouse models (16). Compound heterozygous mice carrying mutations on the same chromosome of both *SMAD4* and *APC*, a colon tumor suppressor, develop more malignant colon tumors than mice with only APC heterozygous mutations, indicating that SMAD4 plays a significant role in the control of malignant progression of colon tumors (16). SMAD4 homozygous knockout mice are embryonic lethal and die between E6.5 and E8.5. SMAD4 heterozygous mice can develop gastric polyposis and tumors in old age. Although some of these tumors exhibited LOH of the *SMAD4* allele, half maintained a normal *SMAD4* allele, suggesting that LOH at the *SMAD4* locus may not be an obligatory event in SMAD4-dependent tumorigenesis (16). SMAD2 has also been shown to be a tumor suppressor in colon cancer (11%) and lung cancer (7%; Refs. 9, 10). In addition, SMAD3 is localized in a hot spot mutation area for breast cancer and a few other types of cancers, and SMAD3 homozygous knockout mice have been reported to develop metastatic colon cancer (9, 10). However, it remains to be determined whether SMAD3 is a tumor suppressor in human cancers.

Intriguingly, *SMAD4* mutations have been found occasionally in conjunction with *TGFBR1* mutations in biliary cancer (15), with *TGFBR2* mutations in colon cancer (17), and with *ACVR1B* mutations in pancreatic cancer (14). This was unexpected, because clonal selection theory would predict that within a neoplastic clone, inactivation of one gene in a given tumor-suppressive pathway provides a selective growth advantage that would abrogate the necessity to inactivate a second gene in the same pathway. The mutual exclusiveness of *p53/MDM2*, of *APC/β-catenin*, and of *p16/CDK4/RB1* genetic alterations has provided support for this theory.

A combined input model has been proposed recently to rationalize the observed coexistence of genetic inactivation of SMAD4 with TGF-β or activin receptor (14). During the early stages of pancreatic tumorigenesis, mutations or expression defects of either the activin or TGF-β receptor can occur in a neoplastic clone, which offers a selective advantage. In this clone, signals of the remaining SMAD4-mediated tumor-suppressive pathways (TGF-β or activin), or perhaps other yet to be identified tumor-suppressive receptor inputs, remain active to partially inhibit growth. During these early stages, SMAD4 somehow provides an essential survival function, thus preventing the emergence of SMAD4-null clones (14). Pancreatic cancer arises from an intraductal precursor, PanIN (pancreatic intraepithelial neoplasia). Loss of SMAD4 expression is restricted to PanIN-3, the most advanced stage before invasion (18). At a very late stage in the intraductal evolution of PanIN, within cells that have acquired multiple genetic defects in cell cycle checkpoints and other regulatory systems, the functional loss of SMAD4 ceases to be detrimental but becomes advantageous. All remaining SMAD4-mediated suppressive signals can then be inactivated by the loss of SMAD4. Subsequently, there is a rapid evolution to the invasive and extremely lethal stage, that of pancreatic carcinoma (14).

How does SMAD4 suppress cancer growth? The tumor suppressor function of SMAD4 is usually studied in the context of TGF-β signaling. The TGF-β pathway is highly mutated in pancreatic cancer, with an overall mutation rate of >80% (15). The individual mutation rates of *TGFBR1*, *TGFBR2*, *SMAD4*, and *p15* are approximately 1, 4, 55, and 30%, respectively (15). Introduction of SMAD4 into human cell lines from pancreas, colon, or breast cancers can restore TGF-β-mediated transcriptional activation, growth inhibition, and apoptosis (11, 12, 19, 20). Together, these observations suggest that the growth-inhibitory function of TGF-β/SMAD4 may be important for SMAD4 tumor-suppressive activity.

On the other hand, several lines of evidence suggest that the tumor-suppressive activity of SMAD4 may not be exclusively dependent on TGF-β or its growth-inhibitory effects. For example, TGF-β growth-inhibitory and transcriptional responses were found to be SMAD4 independent in a SMAD4-

null human pancreatic cancer cell line (21). Murine cell lines with targeted disruption of the *SMAD4* locus have been generated (22). TGF-β responses, including growth arrest (22), induction of the endogenous plasminogen activator inhibitor-1, and other extracellular matrix components, were normal in SMAD4-deficient mouse fibroblasts (22). In contrast, SMAD4 was required for activation of the *Xenopus Mix.2* promoter in response to activin/TGF-β, a reporter gene often used to monitor activin or TGF-β induction. SMAD4 was also involved in the regulation of the Msx homeobox protein family members in response to BMP (22). Additionally, the coexistent homozygous deletion of both the *TGFBR1* and the *p16* genes in one pancreatic cancer and the homozygous deletion of the *TGFBR2* gene and *p16* methylation in another case would suggest that the TGF-β and the p16/RB pathways may mediate functionally distinct tumor-suppressive functions (15).

Moreover, a recent study showed that restoration of SMAD4 to human pancreatic carcinoma cells suppressed tumor formation *in vivo* (23). It did not restore TGF-β sensitivity, which may be explained by the low expression level of endogenous TGF-β type I receptor in that pancreatic carcinoma cell line (23). Unexpectedly, SMAD4 restoration influenced angiogenesis, decreasing expression of VEGF and increasing expression of the angiogenesis inhibitor TSP-1 (23). These findings suggest that in pancreatic adenocarcinoma cells, SMAD4 exerts at least part of its tumor-suppressive function by controlling an angiogenic switch and thus identify VEGF and TSP-1 as tumor-relevant targets of SMAD4. However, it is not yet clear how SMAD4 controls VEGF and TSP-1. The VEGF promoter harbors several potential SMAD-binding sequences. The TSP-1 promoter is devoid of known SMAD-binding elements, suggesting SMAD4 regulation by an indirect mechanism(s). The finding that SMAD4 can inhibit angiogenesis is surprising, because TGF-β can promote angiogenesis (9, 10). Moreover, a recent study shows that the hypoxia-inducible factor-1 and SMAD3 together achieve optimal activation of the VEGF expression in a hypoxia-inducible factor-1 and SMAD-binding sites-dependent manner (24). BMP may also promote angiogenesis because targeted deletion of SMAD5, a mediator of BMP responses, leads to embryonic lethality due to defects in angiogenesis and mesenchymal apoptosis (16). Thus, it will be very interesting to determine whether SMAD4 inhibition of angiogenesis is a general phenomenon in different pancreatic cancer cells.

SMAD4 is a candidate tumor suppressor gene with a strikingly high frequency of gene alterations in pancreatic cancer that suggests a discrete role for SMAD4 in these tumors. Indeed, the report by Tascilar *et al.* (3) indicates that patients undergoing surgical resection for pancreatic adenocarcinoma survive longer if their cancers express SMAD4. Interestingly, a recent study with 456 cases of human breast carcinoma assembled in tissue microarrays indicates that among patients with stage II breast cancer, lack of SMAD2 phosphorylation by TGF-β receptor in the tumor was strongly associated with shorter overall survival (25). Future studies are required to define the molecular mechanisms in each case and to determine whether a related mechanism is involved. The TGF-β superfamily is very complex. It is possible that there are other tumor-suppressive receptors yet to be conclusively demonstrated by the identification of tumor mutations. In any case, SMAD4 expression is correlated with better prognosis of pancreatic ductal carcinoma. The positive impact of SMAD4 on prognosis of this deadly cancer suggests an approach for the development of novel pancreatic cancer therapies in the future.

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