MOLECULAR BIOLOGY OF PANCREATIC CANCER

Z.-W. Xu, H. Friess*, M. W. Büchler
Department of Visceral and Transplantation Surgery, University of Bern, Inselspital CH-3010 Bern, Switzerland

МОЛЕКУЛЯРНАЯ БИОЛОГИЯ РАКА ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ

Ц.-В. Ксу, Х. Фрисс*, М. В. Бюхлер
Отдел брюшной и трансплантационной хирургии Бернского университета, Берн, Швейцария

Pancreatic cancer remains a leading cause of cancer-related death, challenging basic and clinical researchers worldwide. More effective diagnostic and treatment options require better definition of the pathophysiological changes of pancreatic cancer at the molecular level. In this review, we will discuss a number of changes which are commonly present in pancreatic cancer, including 1) molecular alterations in three families of growth factors and their receptors, 2) molecular alterations in tumor suppressor genes, and 3) mutations of the *K-ras* protooncogene. In addition, we will also discuss several apoptosis-related genes which were recently found to have an important role in pancreatic cancer with regard to prognosis and new therapeutic targeting points. A better understanding of the molecular alterations of pancreatic cancer will hopefully lead us to better strategies for diagnosis and treatment of this dismal disease in the future.
Key Words: **pancreatic cancer, gene mutations, tumor suppressor genes, growth factors, receptors, apoptosis.**

Рак поджелудочной железы остается одной из главных причин смерти больных онкологического профиля, бросая вызов ученым всего мира в области фундаментальных и клинических исследований. Для повышения эффективности диагностики и лечения необходимо более глубокое понимание патофизиологических изменений, которые происходят на молекулярном уровне при раке поджелудочной железы. В предлагаемом обзоре обсуждаются различные изменения, характерные для рака поджелудочной железы, в частности: 1) молекулярные изменения в трех семействах факторов роста и их рецепторах; 2) молекулярные изменения в генах–супрессорах опухолевого роста; 3) мутации в протоонкогене *K-ras*. Кроме того, речь пойдет также о некоторых генах, связанных с апоптозом, которые, как было недавно показано, играют важную роль в развитии рака поджелудочной железы. Эти вопросы обсуждаются с точки зрения прогноза и новых "мишеней" для терапии. Можно надеяться, что улучшение понимания молекулярных изменений при раке поджелудочной железы в будущем позволит разработать более эффективные стратегии диагностики и лечения этой опасной болезни.
Ключевые слова: **рак поджелудочной железы, мутации генов, гены–супрессоры опухолевого роста, факторы роста, рецепторы, апоптоз.**

Pancreatic cancer is a devastating disease with a poor prognosis. It is currently the fourth or fifth most common cause of cancer–related death in Western industrialized countries, with over 60,000 deaths in both the European Community and the United States each year [1, 2]. Statistically, the overall five–year survival rate in patients with this disease is less than 1%, and the median survival time is approximately 5–6 months [3]. Due to better diagnostic measures developed in recent years, the number of pancreatic cancer patients seems to have increased. Around 85% of pancreatic cancer patients have unresectable tumors at the time of diagnosis. Additionally, metastatic lesions are frequently found at the time of diagnosis, which is another main reason for failure to effectively treat this disease. Thus, most patients can be offered only palliative surgical options, such as biliary or gastric bypass. Conventional therapeutic cancer strategies such as chemotherapy and radiotherapy have failed to significantly improve the prognosis of advanced pancreatic cancers, due to the cancer's unresponsiveness to cytotoxic agents and radiation. Furthermore, other treatment approaches, such as anti–hormonal modalities or immunotherapy, have also not led to a significant improvement in the overall survival of pancreatic cancer patients [4–7].

The reasons for the biological aggressiveness of pancreatic cancer and the low response to treatment are not clearly defined. However, the achievements of molecular biology over the past two decades have offered us a better understanding of pancreatic cancer, and may contribute to the development of more effective diagnostic and treatment strategies in the future. Many investigations have demonstrated that cancer is a disease with multi–gene alterations. A variety of cancer–promoting genes are involved in the transformation of a normal cell into a cancer cell. These can be divided into two distinct classes: (1) protooncogenes, which might be mutated, amplified or overexpressed and (2) tumor suppressor genes, which might be mutated, deleted, or have both alleles inactivated. It is important to know that many cancer–promoting genes have shown tumor–dependent variations in these genetic alterations. Increa–

Received: January 12, 2000.
* Correspondence. Fax: +41 31 632 9732;
E–mail: helmut.friess@insel.ch

Abbreviations used: CDK — cyclin–dependent kinase; EGF — epidermal growth factor; FGF – fibroblast growth factor; HER — human epidermal growth factor receptor; TGF — transforming growth factor.

sing evidence shows that several inactivated tumor suppressor genes and activated oncogenes are involved in pancreatic carcinogenesis. Furthermore, a variety of growth factors and their receptors are overexpressed in pancreatic cancer [8, 9] which, together with other genetic alterations, promote pancreatic cancer growth and the formation of metastases. In this review we will briefly summarize our present understanding of the molecular changes in pancreatic cancer, focusing mainly on growth factors and their receptors, tumor suppressor genes, oncogenes, and apoptosis–related genes. The original data have been published previously.

GROWTH FACTOR RECEPTORS AND THEIR LIGANDS

The epidermal growth factor receptor (EGF–R) and its ligands. EGF–R, also known as human EGF–receptor type 1 (HER1), is a 170–kDa transmembrane glycoprotein with tyrosine kinase activity. EGF–R shows high amino acid homology with three other transmembrane receptors which are named HER2, HER3 and HER4 [10–12]. They consist of an extracellular ligand–binding domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity [10–12]. EGF–R can be activated by growth factors such as EGF, TGF–α, heparin–binding EGF–like growth factor (HB–EGF), β–cellulin and amphiregulin [13–16]. After binding with the ligand, EGF–R forms homo– and/or heterodimers with HER2, HER3 or HER4 which are activated through autophosphorylation of tyrosine residues [17, 18]. The phosphorylated receptors transmit signals by a variety of intracellular substrates depending on the cell type, the ligand, and the participating EGF receptors [10–12, 18].

With immunohistochemical and *in situ* hybridization techniques, low expression levels of EGF–R, EGF, and TGF–α have been demonstrated in the normal pancreas [19–22]. Generally, EGF–R expression is present in islets, and TGF–α in most ductal cells of the normal pancreas [19–22]. In contrast, EGF–R, TGF–α, and EGF are significantly overexpressed in pancreatic cancer. The concomitant overexpression of both the EGF–R and its ligands, EGF, and/or TGF–α indicates that autocrine and/or paracrine mechanisms of this receptor–ligand system might play a key role in the pathogenesis of pancreatic cancer and in pancreatic cancer growth [19–22]. Furthermore, the concomitant presence of EGF–R and its key ligands in pancreatic cancer cells is associated with increased tumor size, nodal involvement, and a significantly shorter survival time following tumor resection [22].

Early studies on cultured cancer cells showed that increased expression of the EGF–R leads to malignant cell transformation and that the combined presence of EGF and TGF–α further stimulates the proliferation of the transformed cells [13, 23]. The *in vitro* data have now also been confirmed in TGF–α transgenic mice, where a high tumor incidence in conjunction with progressively increased levels of EGF–R are reported [24]. Based on these findings, some new experimental therapies have been designed for use on pancreatic cancer which target the expression of the EGF–R and its ligands. For example, using antisense oligonucleotides against amphiregulin — an extracellular ligand of EGF–R — the growth of pancreatic cancer cell line T_3M_4 was inhibited in a dose–dependent manner [25]. Transfection of a truncated EGF receptor lacking the tyrosine kinase domain into Panc–1 pancreatic cancer cells leads to a marked diminution of EGF receptor–dependent signaling and decreased anchorage–dependent growth of these cancer cells [25]. It also increases the sensitivity of the cancer cells to chemotherapeutic agents. Thus, these observations strongly suggest that pancreatic cancer gets a significant growth advantage through autocrine and/or paracrine mechanisms of the EGF–R/ligand system. New therapeutic strategies designed to abrogate these signaling pathways are promising and seem to offer a new treatment strategy for pancreatic cancer. Furthermore, alterations of HER2 and HER3 have been reported in human pancreatic cancer which might influence the heterodimerization of the EGFR family and thereby tumor pathogenesis and tumor proliferation [26, 27].

The superfamily of transforming growth factor betas (TGF–βs) and their receptors. TGF–βs consist of a large family of polypeptide growth factors that exert different influences on cell growth and differentiation, angiogenesis, extracellular matrix deposition and local immune function [28–30]. The main members characterized in this family include the three mammalian TGF–β isoforms (TGF–β_1, TGF–β_2 and TGF–β_3), activins/inhibins, Mullerian–inhibiting substances and bone morphogenetic proteins (BMPs) [31]. In most epithelial cells, TGF–βs generally act as potent growth inhibitors. However, in cancer, TGF–βs exhibit diverse, even opposite effects on tumor cell growth, depending on the cell type, the culture conditions and the concentration of TGF–β which was used [32].

In human pancreatic cancers, all three TGF–β isoforms are overexpressed in comparison with normal pancreatic tissue, as seen with Northern blot and *in situ* hybridization analysis [33]. Surprisingly, enhanced expression of any of these three TGF–β isoforms in pancreatic cancer tissues was associated with shorter patient survival after tumor resection. Thus, these observations suggest that TGF–βs may function as growth stimulators in human pancreatic cancer, or that the growth–inhibiting effects of TGF–β on pancreatic cancer are altered through disturbances in the downstream signaling pathway.

TGF–βs signal through cell surface TGF–β receptors with serine/threonine kinase activity. After receptor binding, TGF–βs promote the heterodimerization of TGF–β R2 (TβR2) and TGF–β R1 (TβR1). Activated TβR2 then activates TβR1, which will transiently associate with the proteins Smad2 and/or Smad3, members of a recently discovered family of intracellular signaling molecules [34, 35]. After phosphorylation, this complex will be transported into the nucleus and combined with DPC4 (also called Smad4). Smad6 and Smad7, two novel members of this signaling family, can block intracellular signal transduction by forming a stable complex with the intracellular TβR1 domain, resulting in the block–

ing of Smad2/Smad3 activities [36, 37]. Transfection of full–length Smad6 or Smad7 cDNA into pancreatic cancer cell line COLO–357, which is sensitive to TGF–β–indicated growth inhibition, leads to the complete loss of the growth–inhibiting response. Inasmuch as Smad4 is mutated in approximately 50% of pancreatic cancer samples and both Smad6 and Smad7 are overexpressed in pancreatic cancer cells *in vivo*, these observations can explain why TGF–β overexpression is not associated with growth inhibition [38, 39].

Normally, three types of TGF–β receptors are present in the human pancreas [40]. Compared to normal pancreatic tissue, TβR1 and TβR2 are markedly overexpressed in most pancreatic cancer tissues, but similar levels of TβR3 are present in normal and cancer tissues [40, 41]. Furthermore, the advanced stage of pancreatic cancer was found to be associated with the presence of TβR1 or TβR2 (41, 42). These findings suggest that TGF–βs and their receptors are involved in the neoplastic process in pancreatic cancer. Nevertheless, the exact mechanisms and functions of TGF–β signaling in pancreatic cancer are still not clear. It has been proposed that TGF–βs may enhance cancer spread by stimulating angiogenesis, suppressing cancer–directed immune reactions and modulating the composition of the extracellular matrix [41, 42].

FGFs and their receptors. Fibroblast growth factors (FGF) are a family of mitogenic growth factors that are involved in mitogenesis, angiogenesis, chemotaxis, and tissue development and repair [43, 44]. Presently, at least 14 growth factors of this family have been identified. The best known are FGF–1 (acidic FGF) and FGF–2 (basic FGF). Signaling of FGF is mediated by a dual–receptor system via different pathways, including the Ras/Raf/MAP kinase cascade [43, 44].

Currently, four members of the FGF family — FGF–1, FGF–2, FGF–5, and FGF–7 — have been reported to be overexpressed in pancreatic cancers. Concomitant overexpression of FGF–1 and FGF–2 occurs within the cancer cells and is associated with a more advanced tumor stage [45]. FGF–7 overexpression also occurs predominantly in the cancer cells [46], whereas FGF–5 is predominantly overexpressed in stromal fibroblasts which infiltrate and surround the cancer cells and to a lesser extent in the cancer cells themselves [47].

Enhanced FGF receptor expression is also involved in the pathogenesis of human pancreatic cancer [48]. Influencing FGF–dependent signaling in pancreatic cancer cells, for example, by decreasing glypican–1 expression levels with an antisense technique or the introduction of a truncated FGFR–1, which results in a kinase–deficient receptor, abrogates the responsiveness of pancreatic cancer cells to FGF, which raises the possibility that inhibition of FGFR signaling may ultimately also be useful as a therapeutic option in patients with pancreatic cancer [49, 50].

ONCOGENE ALTERATIONS IN PANCREATIC CANCER

K–ras gene mutations in pancreatic cancer. Kirsten (*K*)–*ras* oncogene is located on chromosome 12p13 and encodes a guanine nucleotide binding protein which is involved in signal transduction. It is the most commonly mutated gene in pancreatic cancer (74–100%), although a variety of other oncogenes such as *Her–2/neu* are frequently overexpressed in pancreatic cancer [51]. Mutations of *K–ras* result in ongoing pathway activation leading to disturbed cell proliferation and differentiation. These mutations occur at an early stage in pancreatic carcinogenesis. The mutation sites in *K–ras* genes can be found at codons 12, 13 or 61. For pancreatic cancer, this kind of alteration most often occurs at codon 12, irrespective of whether cultured cell lines or pancreatic cancer tissues are studied [52], and causes alteration of the GDP/GTP binding site, leading to a constitutionally active K–ras protein. *K–ras* gene mutations are detectable with mutation allele specific amplification (MASA) PCR technique in the faeces, plasma, fine–needle aspirates of pancreatic masses, pancreatic juice, and duodenal aspirate of pancreatic cancer patients [53–55]. However, in patients with chronic pancreatitis as well, *K–ras* gene mutations were reported, reducing the diagnostic specificity of *K–ras* mutation analysis in clinical practice [54, 56, 57]. The expression of transduced antisense *K–ras* RNA or antisense oligonucleotides targeting *K–ras* mutation at codon 12 can experimentally suppress the growth of pancreatic cancer cells *in vitro* and *in vivo* [58, 59]. However, the prevalence of this mutation pattern in non–neoplastic and neoplastic cells in the pancreas suggests that *K–ras* mutations might be a carcinogenic factor and might serve as an excellent ancillary diagnostic marker for pancreatic cancer. In addition, therapeutic strategies aimed at abrogating K–ras signaling (farnesyl transferase inhibitors, K–ras antibodies) have been developed and are now being tested in phase I and phase II clinical trials.

TUMOR SUPPRESSOR GENES

p53 tumor suppressor gene. The *p53* gene is a tumor suppressor gene which is mapped on chromosome 17p13. The wild–type p53 protein, encoded by the *p53* gene, is a nuclear phosphoprotein with cancer–inhibiting properties. It functions as a transcription factor to regulate the expression of other genes by binding to specific DNA sequences. So far, the understanding of p53 mediating cellular responses to DNA: damage is focused on two aspects: (a) cell cycle arrest through blocking cell entry into the S from the G_1 phase, and (b) mediation of apoptosis. In cell cycle arrest it is considered that p53 activates p21 protein, a cyclin–dependent kinase inhibitor, which in turn inactivates the kinase required for driving cell cycle progression [60]. In mediation of apoptosis, p53 is considered to promote apoptosis through diverse mechanisms, including transactivation of specific target genes such as *bax, Fas*, and *Death receptor–5* (*DR–5*) genes, and down–regulation of a distinct set of genes including *bcl–2*, *MAP–4* and *IGF1 receptor* genes. It also can induce apoptosis through direct protein–protein interactions by transcription–independent mechanisms [61].

Inactivation of p53 causes a loss of an essential regulator in the cell cycle and an important promoter for apop–

tosis. *p53* point mutations in different sites or loss of heterozygosity (LOH) are frequently observed in pancreatic cancer (40–80%), but not in chronic pancreatitis [62]. Several investigations showed that *p53* mutations are involved in the late events of pancreatic carcinogenesis and are often associated with an advanced tumor stage, the presence of local lymph node metastasis and a shorter survival after tumor resection [63]. Detection of *p53* mutations in combination with *K–ras* mutations in pancreatic juice was considered to be useful for enhancing the genetic diagnosis of pancreatic cancer [64]. There is also evidence that *p53* mutations are associated with chemo–resistance by lowering the threshold of apoptosis [65]. DR–5 expressed in some cancer cells, involving the apoptosis induced by the TNF–related apoptosis–inducing ligand (TRAIL), was found to be upregulated by p53 overexpression or exposure of wild–type p53–containing cells to DNA damaging agents [66]. Clinical studies aiming to restore wild–type p53 function in pancreatic cancers are now under way.

p16 tumor suppressor gene. The *p16* gene is also an important and commonly altered tumor suppressor gene for pancreatic cancer, p16, the protein product of the *p16* gene, normally acts as a potent inhibitor of the cell cycle through blocking the activation of cyclin–dependent kinase 4 (CDK4) by binding to CDK4 subunits and preventing the interaction of CDK4 with cyclin D_1 and the phosphorylation of the Rb protein, finally resulting in cell cycle arrest [67]. In many tumor types, inactivation of *p16* can occur through diverse mechanisms, such as homozygous deletion, point mutations, or hypermethylation in its promoter region, all of which are present in pancreatic cancer [68, 69]. The *p16* gene is inactivated in up to 95% of pancreatic cancers. *p16* mutations are associated with decreased survival and poorer response to postoperative chemotherapy in patients with pancreatic cancer [70]. Hence, it is considered as one of main tumor suppressor genes in pancreatic cancer cases.

DPC4 (Smad4) tumor suppressor gene. In 1996, *DPC4* (deleted in pancreatic cancer, also called *Smad4*) was cloned [71]. It is located on chromosome 18q21 and was suggested to function as a tumor suppressor gene [71]. The long arm fragment of chromosome 18q is considered to be one of the most frequently lost chromosome arms in pancreatic cancer. The *Smad4* gene encodes for a protein called Smad4 which plays a critical role in transmitting growth–suppressive signals from TGFβ. Mutant Smad4 loses its ability to bind Smad2/Smad3, abrogating the TGFβ signaling pathway to the nucleus [72, 73]. Smad4 is inactivated in approximately 50% of pancreatic cancers, in 30% by homozygous deletion and in 20% by LOH combined with an intragenic mutation of the second allele [71]. Interestingly, *Smad4* inactivation seems to be relatively specific for pancreatic cancer, as it occurs in other tumor types with a much lower frequency [74, 75], and is suggested to be an early event in pancreatic ductal tumorigenesis [76].

BRCA2 tumor suppressor gene. The protein product of the *BRCA2* gene is thought to mediate the repair of double–strand breaks in the DNA. Carriers of germline mutations in the *BRCA2* gene are known to be at high risk to develop breast and ovarian cancers. In pancreatic cancer patients, 7% of the *BRCA2* alterations are on chromosome 13q12.3 positions and are frequently combined with LOH [77]. Although the prevalence of *BRCA2* alterations is not high compared with other tumor suppressor genes, a recent investigation in a large series of families revealed strong confirmation of an increased risk of pancreatic cancer in *BRCA2* mutation carriers [78].

KAI–1 gene. KAI–1 is a 29.6–kD membrane glycoprotein originally identified as a metastasis suppressor in prostate cancer [79]. In normal prostate tissue, KAI–1 is more highly expressed than in metastatic prostate cancer cells. To investigate these findings, we analyzed KAI–1 expression levels in pancreatic cancer samples by immunohistochemistry, *in situ* hybridization and Northern blot analysis, and found increased KAI–1 expression in over 80% of primary pancreatic tumors compared with normal pancreatic tissue [80, 81]. However, because tumors with low KAI–1 expression seem more likely to metastasize, our findings indicated that *KAI–1* may function as a potent metastasis suppressor gene in pancreatic cancer [80, 81].

APOPTOSIS–RELATED GENES

bcl–2 and bax genes. Based on studies in recent years, activation of apoptosis has been considered as an important event in tumor biology, involved in tumorigenesis, response to chemotherapy and radiotherapy, and the assessment of the long–term prognosis.

The *bcl–2* gene family has been identified as a key regulator of apoptosis in many cellular systems. This family is commonly divided into both apoptosis–inhibiting (*bcl–2, bcl–*x_L and *Mcl–1*) and apoptosis–promoting (*bax, bak*, and *bad*) genes. The antiapoptotic functions of the *bcl–2* gene product influence the extent of cell survival in both normal and tumor cells by inhibiting different cell death mechanisms following ionizing radiation and chemotherapy [82]. Furthermore, *bcl–2*–overexpressing transgenic mice develop tumors, indicating its important role in tumor development and progression [83]. Clinical experimental data have shown that enhanced expression of *bcl–2* is associated with poorer therapy response and shorter overall survival in patients with prostate cancer and leukemia. Conversely, similar clinical features are associated with reduced expression of *bax* gene in breast cancer patients. In a recent study, 30% and 61% of pancreatic cancer samples exhibited enhanced *bcl–2* and *bax* expression by Northern blot analysis, respectively. By *in situ* hybridization, both mRNA moieties were predominantly expressed in the cancer cells. Analysis of the molecular findings with clinical parameters interestingly revealed that the presence of the apoptosis–promoting *bax* gene product in the pancreatic cancer cells was associated with longer postoperative survival following tumor resection. In contrast, the presence or absence of *bcl–2* in the pancreatic cancer samples had no influence on patient survival or other tumor parameters. These find–

ings suggest that *bax*, but not *bcl–2*, influences the biological growth behavior of pancreatic cancer *in vivo* and upregulation of *bax* correlates with a better prognosis in pancreatic cancer patients [84].

bcl–x_L gene. The *bcl–x* gene is spliced into two mRNA transcripts — *bcl–x_L* and *bcl–x_S*. *bcl–x_L* is the longer transcript of the *bcl–x* gene. It functions as a broad antiapoptotic factor, extending both normal and tumor cell survival. Inasmuch as tumor cell death induced by chemotherapeutic agents and radiotherapy is mainly mediated by activation of apoptosis, the activation of apoptotic genes in conjunction with the inactivation of anti–apoptotic genes seems to be critical for tumor response to these treatment modalities [85].

In pancreatic cancer, expression of the *bcl–x_L* gene is enhanced, and its expression is associated with short patient survival [86]. Using a specific antisense oligonucleotide target on *bcl–x_L* mRNA, we found that cultured pancreatic cancer cells which exhibit high *bct–x_L* expression died by induction of apoptosis. This was confirmed by DAPI staining and FACS analysis. *bcl–x_L* antisense oligonudeotides also increased the growth inhibitory actions of the chemotherapeutic agent gemcitabine, up to 25%. These findings suggest that *bcl–x_L* in pancreatic cancer *in vivo* plays an important role in apoptosis modulation, and inhibition of *bcl–x_L* expression may be a promising target for gene therapy and a sensitivity–increased chemotherapy in pancreatic cancer.

CONCLUSION

In the past years, the development of modern molecular and cellular biology techniques has significantly contributed to an increased understanding of the pathophysiological changes in pancreatic cancer. Discoveries of alterations in genes, signaling of growth factors, and promotion or inhibition of apoptosis have given us a better understanding of the carcinogenesis of pancreatic cancer. Some new technologies, such as gene chip analysis, will facilitate a more rapid and extensive identification of gene alterations in the near future. Although these achievements are promising, many genetic alterations are still unknown. The future task will be to develop new diagnostic and therapeutic tools based on this knowledge. There is no doubt that effective therapeutic strategies for pancreatic cancer will apply our present molecular knowledge of this disease and will require further discoveries in the future.

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