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Original Paper

Cholecystokinin Receptors in Human Pancreatic Cancer Cell Lines

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Recent studies have suggested that cholecystokinin (CCK) receptors may play a role in the development and growth of pancreatic cancers. We detected the expression of mRNA encoding CCK-A and CCK-B receptors in eight human pancreatic tumour cell lines using reverse transcription-polymerase chain reaction (RT-PCR), but not by RNase protection assays. The K-*ras* gene, which can be activated by G-coupled protein receptors such as CCK receptors, was mutated in codon 12 in five of the cell lines. In addition, Mia PaCa-2 pancreatic cancer cells did not respond to CCK or gastrin in cell proliferation or focal adhesion kinase (FAK) phosphorylation assays. In contrast, mouse NIH3T3 fibroblasts transfected with human CCK-B receptor (NIH3T3CCK-BR) showed increased proliferation and phosphorylation to the peptides. Also, radioligand binding studies indicated that Mia PaCa-2 cells had approximately 12.5-fold less CCK-B receptors than NIH3T3CCK-BR. Our results suggest that in Mia PaCa-2 cells, CCK receptors may not play a crucial role in supporting cell growth. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: human pancreatic cancer, cell lines, cholecystokinin receptors, focal adhesion kinase, K-*ras*
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INTRODUCTION

CANCER OF the pancreas is the fifth leading cause of death from malignant disease in Western society. More than 90% of patients die within a year of diagnosis, and the 5-year survival rate is less than 1% [1]. The underlying molecular pathology of pancreatic carcinoma is unknown, although over 90% of pancreatic carcinomas investigated have mutations which activate oncogenes, predominantly codon 12 of K-*ras* [2]. In addition, approximately 50% of pancreatic carcinomas have transition-type missense mutations within highly conserved regions of the *p53* tumour suppressor gene [3]. Some pancreatic cancers and pancreatic tumour cell lines have deletions or mutations in the *p16/MTS-1* tumour suppressor gene [4]. Loss or mutation of the *DPC4* gene (deleted in pancreatic cancer, locus 4), a candidate tumour suppressor gene [5], may also contribute to the aggressive nature of this cancer.

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Pancreatic cancers are resistant to radiation and chemotherapy and, therefore, alternative treatments, including the use of specific growth factor receptor antagonists, such as loxiglumide, are being investigated, There are conflicting results regarding the effects of the peptide hormones cholecystokinin (CCK) and gastrin on pancreatic tumour growth, with both stimulatory and inhibitory effects on cell proliferation reported [6–8].

Two CCK receptor-types, CCK-A and CCK-B/gastrin, have been identified using binding affinity studies of sulphated and non-sulphated forms of CCK, gastrin and specific antagonists [9–11]. The recent cloning and mapping of the CCK-A and CCK-B receptors [12, 13] has allowed the investigation of their mRNA expression in various tissues and cell lines. The hydrophobicity plots of the encoded proteins suggest a seven-transmembrane structure typical of guanine nucleotide G-coupled receptor proteins.

Phosphorylation of focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase [14], is an early event following stimulation of CCK and other G-coupled receptors by agonists

which may in turn regulate downstream signalling pathways. Recently, Taniguchi and co-workers [15] demonstrated FAK phosphorylation to be an early response to sulphated CCK-8 (sCCK-8) in mouse NIH3T3 fibroblast cells transfected with the human CCK-B receptor (NIH3T3CCK-BR cells).

In this study, we investigated the expression of CCK-A and CCK-B/gastrin receptors in eight human pancreatic tumour cell lines (Mia PaCa-2, Capan-1, Capan-2, AsPc-1, BxPc-3, Hs766T, Panc-1 and KPan) by reverse transcription-polymerase chain reaction (RT-PCR) and RNase protection assays (RNP), Since CCK receptors are G-coupled protein receptors [12, 13] and their activation has the potential to modulate growth via activation of K-*ras*, the K-*ras* status was also investigated. Exons 1 and 2 of K-*ras*, which encompass codons 12, 13 and 64, respectively, were amplified by RT-PCR and analysed by single strand conformational polymorphism (SSCP) and samples showing band shifts were sequenced. Furthermore, the effects of sCCK-8 and non-sulphated gastrin 17 (nsG-17) on cell proliferation and FAK phosphorylation with the human pancreatic tumour cell line Mia PaCa-2, and the mouse NIH3T3CCK-BR fibroblast cell lines, were compared. The receptor characteristics of these two cell lines were also compared using a specific CCK-B receptor antagonist, [^{3}H]-PD140,376 in radioligand binding studies.

MATERIALS AND METHODS

Cell culture

Seven human pancreatic cancer cell lines (Capan-1, Capan-2, AsPc-1, BxPc-3, Hs766T, Mia PaCa-2 and Panc-1) were purchased from ATTC (Rockville, U.S.A.), while KPan and Jurkatts T cell lymphoma were a gift from Dr D. Darling (King's College, London, U.K.). NIH3T3 and NIH3T3CCK-BR cells were kindly provided by T. Matsui [15].

The cell lines were grown in a sterile incubator at 37°C in 10% carbon dioxide, in either DMEM or RPMI 1640 containing 10% fetal calf serum, 100 U/ml penicillin and streptomycin in 75 cm^2 flasks until 70% confluence was reached, For RNA and DNA extractions, cells were harvested from culture flasks using trypsin and collected by centrifugation at 130 *g*.

Cell proliferation assay

Mia PaCa-2 and NIH3T3CCK-BR cells (0.3×10^4 in DMEM/10% fetal calf serum were plated on to 24 well plates ($\times$10 plates), incubated for 5 days and then for a further 4 days in serum-free medium. Cells were stimulated for 10 days with sCCK-8, nsG-17 (Cambridge Research Bioscience, Cambridge, U.K., 10 pM–100 nN) or 10% fetal calf serum, with daily replacement with fresh identical medium. Cells were harvested daily and counted using trypan blue exclusion.

Analysis of CCK-A and CCK-B receptor status

RNA extraction. Total RNA was extracted from each of the pancreatic tumour cell lines by the method of Chomczynski and Sacchi [16] utilising RNAzol (Biogenesis, Bournemouth, U.K.). The quality of the RNA was confirmed by agarose gel electrophoresis in formaldehyde, distinct 28s and 18s ribosomal RNA bands being used as a measure of the integrity of all RNA species.

RT-PCR of CCK-A and CCK-B receptors. Tumour cell RNA (10 μg) was reverse transcribed into first strand complementary DNA (cDNA) using the Superscript RNase H-Reverse Transcriptase kit (GIBCO-BRL, Paisley, U.K.). RT-PCR was performed on first strand cDNA (2.0 μl) using 35 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C. The PCR products were electrophoresed on a 1% agarose Tris-boric acid EDTA gel containing ethidium bromide (0.1 μg/ml).

The PCR amplification primers for the *CCK*-A and *CCK*-B genes were chosen using the oligo primer analysis software [17] distributed by Medprobe (Oslo, Norway) and were based on the gene sequences published by de Weerth and colleagues [12] and Pisegna and associates [13]. CCK-A primer pair, forward: 5′CCTACGACACCGCCTCCGC3′, reverse 5′TCCGTTCTTTCTTCTCTGCCTCCT3′. CCK-B primer pair, forward: 5′ACCCCAACGACAGGAAAA-GGT3′, reverse primer: 5′TTTGGGAAGGAAGGAGA-GGGC3′.

Cloning and sequencing of the CCK-A and CCK-B receptor PCR products. CCK-A and CCK-B receptor PCR products were cloned using a pCRII TA cloning kit according to the manufacturer's instructions (In Vitrogen, Carlsbad, Canada) and recombinant plasmid DNA was prepared and sequenced using the DNA sequencing kit from Pharmacia (Milton Keynes, U.K.).

RNP. The level of expression of these receptors was determined by RNP using the RPA II kit (AMS Biotechnology, Burford, U.K.). Antisense and sense probes for CCK-A and CCK-B were constructed and high specific activity radiolabelled RNA probes synthesised (at least 4×10^8 cpm/μg). Sample RNA (50 μg) was hybridised with a radioactively labelled RNA probe (600 pg, 42°C, 12 h). The remaining radiolabelled single strand RNA was electrophoresed on a 6% denaturing polyacrylamide gel followed by autoradiography to determine the size and intensity of radiolabelled RNA.

*Analysis of K-*ras *status*

Genomic DNA (50 ng) extracted from the pancreatic cancer cells was PCR amplified with two pairs of K-*ras* primers [4, 18] for exons 1 and 2 (Oswell, Southampton, U.K.). The PCR products were electrophoresed on an agarose gel to confirm the DNA fragment size of 120 and 111 bp, respectively.

For PCR-SSCP analysis, the PCR amplification was carried out with [^{32}P]-deoxy-cytidine triphosphate (2 μCi, 3000 Ci/mmol; Amersham, U.K.). A portion of the PCR product was heated at 95°C for 5 min in loading buffer, rapidly cooled on ice and electrophoresed on a polyacrylamide gel, transferred to Whatman paper and autoradiographed.

FAK phosphorylation analysis

NIH3T3CCK-BR and Mia PaCa-2 cells (1×10^6 per petri dish) were grown in 10% fetal calf serum for 48 h and then in serum-free DMEM for 24 h. The cells were stimulated for 5 min with either sCCK-8 (10–500 nM), nsG-17 (10–500 nM), 10% fetal calf serum or phosphate buffered saline (PBS) as a control and FAK phosphorylation was assayed as previously described [14] using protein A beads (Sigma, Poole, U.K.), rabbit antimouse IgG (DAKO, High Wycombe, U.K.). FAK antibody (Affiniti Research Products Ltd, Exeter, U.K.), antiphosphotyrosine antibody (PY20, Affiniti), horseradish peroxidase (DAKO) and enhanced chemiluminescence assay (ECL; Amersham Radiochemical Company, Amersham, U.K.).

Radioligand binding assay

Mia PaCa-2 (1×10^9) and NIH3T3CCK-BR cells (2×10^9) were harvested with versene (Sigma), pelleted at 800 *g* and

resuspended in Hepes–NaOH buffer (10 ml, pH 7.2 at 21°C). Cells were aliquoted in triplicate at increasing concentrations (3×10^4–3×10^7 cells in 400 µl) and incubated with [^{3}H]-PD140,376 (50 µl of 1 nM, 51.6 Ci/mMol, Amersham Radiochemical Company), a highly selective CCK-B antagonist [19], for 150 min at 21 ± 3°C. Total and non-specific binding of [^{3}H]-PD140,376 was defined in the absence or presence of 50 µl of 10 µM L-365,260 (CCK-B receptor antagonist, pK_ICCK-B~8.69 [20]), respectively. Assays were terminated by rapid filtration through presoaked Whatman GF/B filters and washed (3×3 ml) with 50 mM Tris–HCl (pH 7.4) at 4°C using a Brandell cell harvester and bound radioactivity determined in a Beckman LS6500 liquid scintillation counter.

RESULTS

CCK receptor status

RT-PCR. The two sets of gene specific primers were effective in amplifying either a 340 bp fragment exclusively from the CCK-A receptor or a 430 bp fragment exclusively from the CCK-B receptor. A comparison of the DNA sequences from the PCR products with published data [12, 13] confirmed that the amplified PCR products were fragments of the *CCK-A* and *CCK-B*/gastrin receptor gene. The presence of CCK-A and CCK-B/gastrin receptor mRNA was detected in all eight pancreatic tumour cell lines investigated (Capan-1, AsPc-1, BxPc-3, Hs766T, Mia PaCa-2, Panc-1, KPan and Capan-2, Figure 1). Jurkatt's T cell lymphoma cell line, which is known to express both CCK receptor type, was utilised as a positive control for the expression of CCK-A and CCK-B receptors.

RNP. RNP was used to analyse natural CCK-A and CCK-B receptor expression in total RNA (50 µg) extracted from each of the pancreatic tumour cell lines. These failed to detect CCK-A and CCK-B receptor expression in any of the pancreatic tumour lines investigated (data not shown). A clear band of the predicted size for both protected CCK-A (340 bp) and CCK-B (430 bp) receptor m-RNA was found in human gall bladder and stomach mucosa (data not shown).

K-ras *status.* PCR amplification of K-*ras* exon 1 and exon 2 resulted in fragments of 120 bp and 111 bp, respectively. SSCP data showed genetic variability in exon 1 of the K-*ras* gene in cell lines Capan-1, AsPc-1, Mia PaCa-2, Panc-1 and Kpan when compared with normal pancreas. Cloning and sequencing of the K-*ras* PCR products showed that in two of these cell lines, Capan- 1 and Mia PaCa-2, there was a point mutation resulting in amino acid substitution from the wild-type glycine to a valine at codon 12. The remaining three cell lines, AsPc-1, Panc-1 and Kpan showed a point mutation resulting in amino acid substitution from glycine to aspartate (Table 1). Mutations were not detected at the alternative activating site of codon 61 in exon 2 in K-*ras* in any of the cell lines investigated.

Table 1. *Summary of K*-ras *mutations in human pancreatic cancer cell lines*

Cell line	K-*ras* Exon 1 codon 12	K-*ras* Exon 2 codon 61
Capan-1*	G**T**T	CAA
AsPc-1*	G**A**T	CAA
BxPc-3	GGT	CAA
Hs766T	GGT	CAA
Mia PaCa-2*	G**T**T	CAA
Panc-1*	G**A**T	CAA
Kpan*	G**A**T	CAA

The point mutations in exon 1 at codon 12 are underlined. GGT, glycine; GAT, aspartate; GTT, valine; CAA, glutamine. *Tumour cell lines possess a point mutation in codon 12 of the K-*ras* gene. No mutations were found in codon 61.

Cell proliferation

Cell proliferation of the Mia PaCa-2 cell line maintained in serum-free medium did not increase in the presence of either sCCK-8 or nsG-17 (10 pM to 100 nM range). However, 10% fetal calf serum stimulated the growth of Mia PaCa-2 over 10 days by up to 10-fold. In contrast, sCCK-8, nsG-17 or 10% fetal calf serum stimulated the proliferation of NIH3T3CCK-BR cells, transfected with CCK-B receptors, grown in serum-free medium by a maximum of 2-, 1.5- and 8-fold, respectively.

FAK phosphorylation

Mia PaCa-2 cells, growth arrested in serum-free medium, were found to contain phosphorylated FAK and there was no further detectable increase in these levels of phosphorylated FAK following the addition of either sCCK-8 or nsG-17 (10–500 nM) (Figure 2). In contrast, phosphorylated FAK was not detected in NIH3T3CCK-BR cells grown in serum-free medium. However, the addition of either sCCK-8 or nsG-17 (100 nM) promoted FAK phosphorylation as shown by tyrosine phosphorylation of the 125 kDa FAK (Figure 2).

Radioligand binding studies

The total and non-specific binding of [^{3}H]-PD140,376 increased with increasing number of both Mia PaCa-2 and

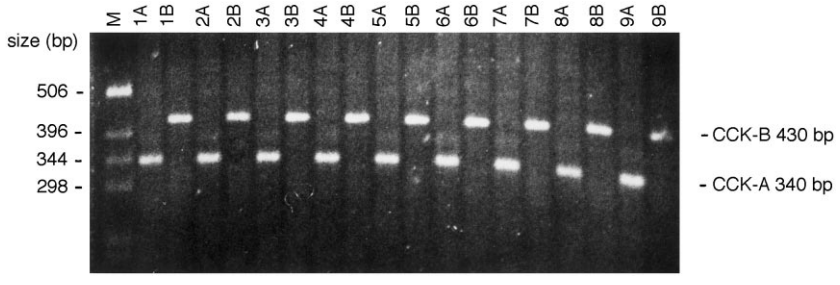

Figure 1. Autoradiograph of an agarose gel electrophoresis separation of reverse transcription-polymerase chain reaction (RT-PCR) products of CCK-A and CCK-B receptor mRNA in human pancreatic cancer cell lines. Lanes 1A, 1B, Capan-1; 2A, 2B, AsPc-1; 3A, 3B, BxPc-3; 4A, 4B, Hs776T; 5A, 5B, Mia PaCa-2; 6A, 6B, Panc-1; 7A, 7B, KPan; 8A, 8B, Capan-2; 9A, 9B, T-cell line; m, molecular markers. The sizes of the amplified PCR receptor fragment products were 340 bp for the CCK-A (A) and 430 bp for CCK-B (B). A T-cell lymphoma cell line was used as a positive control in this assay.

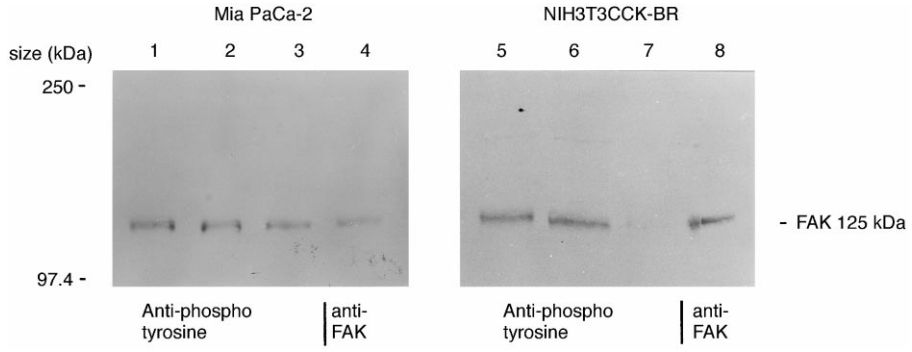

Figure 2. Autoradiograph of a Western blot of a 6.5% polyacrylamide gel in focal adhesion kinase (FAK) phosphorylation studies in Mia PaCa-2 cells and NIH3T3CCK-BR cells. Mia PaCa-2 cells: lane 1, sCCK-8 (500 nM); lane 2, untreated; lane 3, positive control (endothelial cell lysate); lane 4, FAK control cell lysate. NIH3T3CCK-BR cells: lane 5, sCCK-8 (100 nM); lane 6, nsG-17 (100 nM); lane 7, untreated; lane 8, FAK control cell lysate. Lanes 1–3, 5–7 were immunoblotted with antiphosphotyrosine antibody and lanes 4 and 8 with anti-FAK antibody. FAK was constitutively phosphorylated in Mia PaCa-2 cells and not further promoted by sCCK-8. FAK tyrosine phosphorylation was induced by sCCK-8 and nsG-17 in NIH3T3CCK-BR cells.

NIH3T3CCK-BR cells. The specific binding of [^{3}H]-PD140,376 increased linearly, up to 3×10^7 Mia PaCa-2 cells and 3×10^6 NIH3T3CCK-BR cells (Figure 3). At cell numbers where approximately 10% of the added [^{3}H]-PD140,376 was bound (1×10^7 Mia PaCa-2 cells and 1×10^6 NIH3T3CCK-BR cells), the specific binding for these cells was 64 and 59%, respectively, At these cell numbers, approximately 1.25-fold more specific binding was obtained for the NIH3T3CCK-BR cells than for the Mia PaCa-2 cells. If [^{3}H]-PD140,376 has the same affinity for CCK-B receptors in both cell types, then the data suggest that the CCK-B receptor density is 12.5-fold higher in NIH3T3CCK-BR than in Mia PaCa-2 cells.

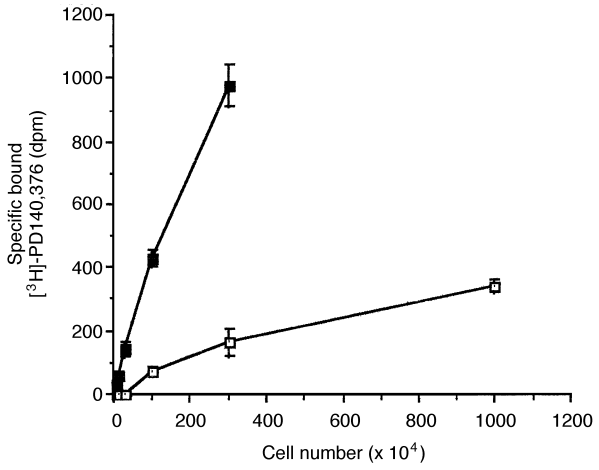

Figure 3. [^{3}H]-PD140,376 binding to Mia PaCa-2 and NIH3T3CCK-BR cells. Mia PaCa-2 (—□—), NIH3T3CCK-BR cells (—■—). The experiment was carried out in triplicate. NIH3T3CCK-BR cells had 12.5 times the affinity for [^{3}H]-PD140,376 compared with Mia PaCa-2 cells.

DISCUSSION

The observed trophic effects of CCK on the normal pancreas *in vivo* [21] and the stimulation of mucosal cell growth in the acid-secreting region of the stomach [22], indicate that CCK/gastrin could stimulate pancreatic cancer cell growth, However, studies investigating the trophic effects of exogenous CCK have led to conflicting results. CCK has been shown to stimulate the growth of KP-1N cells [6] and to increase uptake of [^{3}H]-thymidine in the PC-F3 cell line [8]. Trophic responses to CCK-8 or CCK-9 analogues have been reported for Mia PaCa-2, BxPc-3, Capan-2 and Panc-1 cells [23]. In contrast, others have shown no effect of these peptides on pancreatic tumour cell growth and some studies have even shown inhibition of cell growth [7, 8, 24].

In this investigation on the analysis of mRNA encoding the two receptor subtypes, CCK-A and CCK-B, we were unable to detect transcripts for these receptors using Northern blot analysis of RNA extracted from the pancreatic cell lines. RT-PCR and RNP were, therefore, carried out. Because of the sensitivity of RT-PCR, we were able to detect mRNA, encoding CCK-A and CCK-B receptors in all of the cell lines investigated. RNP using high specific activity radiolabelled probes, failed to detect naturally expressed m-RNA of CCK-A or CCK-B receptors in any of the cell lines. Theoretically, RT-PCR requires only a few molecules of mRNA, while RNP requires at least 5 fg of natural CCK receptor mRNA molecules for detection.

Recently, it has been suggested that stimulation of the gastrin/CCK-B receptor promotes mitogen-activated protein kinase (MAPK) activation in a ras-dependent manner [25]. However, more than 90% of pancreatic carcinomas studied have mutations which disturb the nucleotide binding domain and result in an activated K-*ras* p21 protein bound to GTP [2]. This activation produces increased signal transmission to downstream effectors such as MAPK [26] and could lead to cellular responses, such as growth stimulation, independently of CCK receptor activation. In our study, five of the seven pancreatic tumour cell lines studied possessed a point mutation in codon 12 of the K-*ras* gene. These results are supported by the work of others, who also showed that two of these cell lines (Panc-1 and Mia PaCa-2) have point mutations at codon 12 of K-*ras* as a result of a GGT (glycine) mutation to GAT (aspartate) or GTT (valine), respectively [20, 27]. These results are consistent with other work where it has been shown that 90% of K-*ras* mutations in pancreatic cancer are located at codon 12 [2].

Focusing on the Mia PaCa-2 cell line, we found no proliferative response of these cells to sCCK-8 or nsG-17, suggesting that the receptors maybe absent or at very low levels at the cell surface. Alternatively, the activation of these receptors alone may be insufficient to support full cell growth. Since mRNA levels alone may not provide the true

indication of functional receptor protein levels at the cell membrane, CCK-B receptors in Mia PaCa-2 cells were studied using the receptor antagonist [^{3}H]PD140,376. The binding results obtained were in agreement with the RNP and growth studies which suggested that the CCK-B receptor in Mia PaCa-2 cells was present at a low level compared with those levels present in NIH3T3CCK-BR cells.

The cloning and mapping of the CCK-A and CCK-B receptors [12, 13] have revealed that these receptors are pertussis toxin insensitive, G-coupled proteins. Stimulation of the human CCK-B receptors in NIH3T3CCK-BR cells leads to activated phospholipase C and phosphorylation of several protein species, including MAPK [26] and FAK [15]. Since FAK phosphorylation is an early response of cells to CCK receptor stimulation [14], we also investigated CCK receptor activation in Mia PaCa-2 cells by studying FAK phosphorylation levels following the addition of sCCK-8 and nsG-17. We found that FAK in unstimulated Mia PaCa-2 cells was already phosphorylated and that further increases in phosphorylation by the addition of sCCK-8 and nsG-17 did not occur. These results suggest that alternative signalling pathways may exist that can maximally activate FAK phosphorylation in Mia PaCa-2 cells independent of CCK.

In conclusion, our findings of low natural levels of receptor mRNA using RNP and the presence of activated K-*ras* in many pancreatic cancer cell lines suggests that the activation of CCK receptors may not be required to support growth of pancreatic tumour cell lines. Moreover, others have shown that 90% of the human pancreatic carcinomas investigated also have activated K-*ras*, which could circumvent the need for CCK receptor stimulation. These findings suggest that the development of CCK antagonists may not provide an alternative therapy for this disease.

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