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DISTRIBUTION OF CCK-B RECEPTOR GENOTYPE BETWEEN PANCREATIC CANCER PATIENTS AND CONTROLS AND ITS IMPACT ON SURVIVAL

Jill P. Smith, MD[1,2], **David C. Whitcomb, MD, PhD**[3], **Gail L. Matters, PhD**[4], **Randall E. Brand, MD**[3], **Jiangang Liao, PhD**[5], **Yu-Jing Huang, PhD**[6], and **Marsha L. Frazier, PhD**[6]

[1]Dept. of Medicine, Georgetown University, Washington, DC, USA

[2]Dept. of Medicine, Pennsylvania State University, College of Medicine, Hershey, PA

[3]Department of Medicine, University of Pittsburgh & UPMC, Pittsburgh, PA

[4]Dept. of Biochemistry & Molecular Biology, Pennsylvania State University, College of Medicine, Hershey, PA

[5]Public Health Sciences, Pennsylvania State University, College of Medicine, Hershey, PA

[6]Department of Epidemiology, University of Texas at MD Anderson Cancer Center, Houston, TX, USA

Abstract

Objective—Cholecystokinin (CCK) and gastrin stimulate growth of pancreatic cancer through the CCK-B receptor (CCK-BR). A splice variant of the CCK-BR that results from a single nucleotide polymorphism (SNP) has been identified. Since the splice variant receptor has an extended 3rd intracellular loop, an area involved in cell signaling and growth, we hypothesized that this genetic variant could contribute to the poor prognosis and short survival of this malignancy.

Methods—DNA from 931 patients with pancreatic cancer was evaluated for the SNP (C >A; rs1800843) in the CCK-BR gene. For statistical analysis, the Fisher's exact test was used to compare the genotype and allele frequency between the cancer cohort and normal controls and the dependence of genotype on factors, such as stage of disease and age, was analyzed using Cox's proportional hazard models.

Results—Compared to the normal cohort, the frequency of the A-allele in pancreatic cancer subjects was increased (p=0.01123; OR=2.283). Even after adjustment for stage of disease, survival of subjects with the minor allele was significantly shorter than those with the wild-genotype (HR=1.83; $p = 3.11 \times 10^{-11}$).

Correspondence to: Jill P. Smith, MD, Georgetown University, 3800 Reservoir Rd. NW, 2 Main, Washington DC 20007, Phone: 202-444-8541, Fax 877-444-3957, jps261@georgetown.edu.

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Conclusion—The CCK-BR SNP predicts survival and should be studied as a candidate genetic biomarker for those at risk for pancreatic cancer.

Keywords

SNP; single nucleotide polymorphism; cholecystokinin; Receptor; CCK; mutation; neuroendocrine tumors

INTRODUCTION

In spite of the success in diagnosis and treatment of cancers over the years, no improvement has occurred in the survival of pancreatic cancer[1] which carries the poorest prognosis of all gastrointestinal malignancies.[2] In fact, the 5-year survival rate for pancreatic cancer is about 5–6%, the lowest of any cancer[3] and most treatment becomes palliative.[4,5] A recent review regarding trends for cancer incidence and survival, estimates that by the year 2030, pancreatic cancer will surpass colon and breast cancer to become one of the top two killers of cancer-related deaths in the USA[6]. Reasons for the poor survival rates reported for pancreatic cancer include both the inability to diagnose this disease in the early stages and also the aggressive nature of this malignancy.[7] Although surgical resection offers a potential chance for a cure for pancreatic cancer if detected early,[8] over 90% of the subjects have advanced disease at the time of presentation.[9] Unfortunately, even in the absence of macroscopic disease during surgery and with combined administration of adjuvant chemotherapy, only 20.7% of surgically resected patients survive 5 years.[10] Recent studies suggest that circulating cancer epithelial cells are detected in the blood prior to the radiographic detection of pancreatic cancers[11] indicating the need for novel biomarkers that can detect those subjects at risk rather than biomarkers for cancer detection, a time when it is probably too late.

In cancer research, breakthroughs for improving therapy and survival have all come through a better understanding of the genetics and the biology of the particular malignancy including the identification of tumor-specific receptors and /or mutated genes associated with the cancer. Scientists have been searching for biomarkers or genetic mutations that may enhance earlier detection of pancreatic cancer, identification of high risk populations, and improve our understanding of the aggressive nature of this malignancy. The gastrointestinal peptides gastrin[12] and cholecystokinin (CCK)[13,14] have been shown to stimulate growth of pancreatic cancer, and this research has recently been reviewed.[15] Although pancreatic cancer tissue may have receptor RNA for both the CCK-A receptor (CCK-AR) and the CCK-B receptor (CCK-BR) types,[16,17] selective receptor antagonist experiments have demonstrated that the growth effects of these peptides are mediated through the CCK-BR and not the CCK-AR.[12,18] Similarly, Reubi et al.,[19,20] studied CCK receptors in pancreatic neuroendocrine tissues and found that they possess the CCK-BR receptor type and not the CCK-AR. We have characterized the CCK-BR binding affinity and capacity in human ductal pancreatic cancer and found that the expression of this receptor is ubiquitous in this malignancy, and the receptor number is markedly over expressed compared to normal human pancreas tissue.[21] Indeed, down regulation of the CCK-BR gene significantly inhibits growth of pancreatic cancer.[22] Furthermore, the expression of the CCK-BR has recently been

identified in early human pancreatic epithelial neoplasms (PanINs) and blockade of this receptor with a CCK receptor antagonist blocks PanIN progression in the Kras transgenic mouse model.[23] These investigations support the role of the CCK-BR in pancreatic carcinogenesis.

Some human pancreatic cancers have a splice variant of the CCK-BR that occurs due to alternative splicing of the 4th intron of the CCK-BR gene[24,25] and subsequent translation of the intron leads to the addition of 69 amino acids to the 3rd intracellular loop of the receptor. The CCK-BR is a classic GTP-protein coupled receptor (GPCR).[26] After receptor activation, signal transduction is triggered through a cascade of events initiated by GTP-proteins that interact with this 3rd intracellular loop resulting in growth and /or proliferation, differentiation, migration, angiogenesis, and survival.[27] We raised a monoclonal antibody to the extended segment of the splice variant receptor and found that about a third of pancreatic cancers express the splice variant and reacted to the antibody.[28] DNA sequencing of the CCK-BR revealed a single nucleotide polymorphism (SNP) (C>A) in position 32 of the 4th intron corresponding to rs1800843 that was responsible for the mis-splicing. Only tissues containing A-allele SNP expressed the splice variant receptor and reacted to the monoclonal antibody by immunohistochemical stains.[28] We herein hypothesize that since the CCK-BR SNP alters the 3rd intracellular loop, an area involved with cellular proliferation, the presence of this SNP could be associated with the aggressive nature or poor survival observed in pancreatic cancer. The aims of the present investigation were to compare the CCK-BR genotype distribution in a large cohort of pancreatic cancer patients to controls without cancer, and evaluate the impact of the minor allele SNP on patient survival.

MATERIALS AND METHODS

Study Participants

Participants were drawn from three cohort studies including the prospective pancreatic cancer cohort from Pennsylvania State University (PSU-G),[28] the PAGER (Pancreatic Adenocarcinoma Gene Environment Risk) cohort from University of Pittsburgh Medical Center, and the TEXGEN consortium cohort from University of Texas MD Anderson Medical Center. Cases were identified as pancreatic ductal adenocarcinoma according to ICD-O-3 codes (C250-C253) or ICD-9 codes (157.0 to 157.3) and / or histologic confirmation by surgical pathology reports. Subjects with acinar cell, or squamous cell tumors of the pancreas were excluded. However, subjects with islet cell or neuroendocrine tumors of the pancreas were evaluated separately for the rs1800843 SNP of the CCK-BR. Both tissue and blood specimens were available from the PSU-G cohort as well as buccal mucosal swabs. The PAGER and TEXGEN cohorts included blood specimens from patients with the confirmed histologic diagnosis of pancreatic cancer seen in the outpatient clinics (surgical, gastroenterology, and oncology) at the prospective institutions.

Normal control subjects without cancer were included procured pancreas specimens from deceased donors obtained through an approved protocol from the Gift of Life, Philadelphia, PA. Additional normal controls included specimens from subjects undergoing surgery for a benign pancreatic condition (i.e., pancreatitis or pseudocyst). Each participating center obtained informed consent from the volunteers and Institutional Review Board approval.

Family members signed informed consents at the time of procurement of tissues for transplantation from deceased donors and these tissues were de-identified before use.

Each cohort provided data regarding age at diagnosis, gender, and reported ethnicity. For the survival analysis, each cohort provided the survival time for each participant defined as the time from disease diagnosis to either death or the last censored visit or last contact. Stage of disease at diagnosis was also reported for each subject defined as Stage 1: local disease resectable; Stage 2: borderline resectable; Stage 3 –locally spread to vessels or lymph nodes; and Stage 4 – metastatic to distant sites.

DNA extraction and Sequencing

DNA was extracted from surgical specimens from patients with pancreatic cancer, from benign pancreatic tissue controls, and from normal pancreas tissue donated by procurement. In order to exclude any differences from tumor heterogeneity from the cancer patients and to determine if the variant was germline (not somatic), patient DNA was also obtained by a buccal mucosa swab or blood from these subjects when possible.

Tissue DNA was isolated from approximately 20 mg of surgical specimens using the DNeasy kit according to the manufacturer's instructions (Qiagen). DNA was extracted from patient blood with Autopure LS automated DNA purification instrument (Gentra Systems, Minneapolis, MN). DNA genotyping was done using a TaqMan SNP Genotyping Assay (C__22273290_10) (Applied Biosystems) for the single nucleotide polymorphism (SNP) rs1800843 allele as either C or A.

Statistical analysis

We tested that the genetic variant (SNP) rs1800843 that was studied followed Hardy-Weinberg equilibrium among controls. Genotype and allelic frequency were compared between the cancer cohort and the normal controls using Fisher's exact test and the odds ratio. The effect of various tumor characteristics (stage of disease) and patient demographics (genotype, age at diagnosis, gender) as predictors on survival were assessed using the Cox proportional hazards regression individually and in combination with the genotype using SAS Proc PHREG. The median survival times (MST) in months for the cancer cohort were calculated for each genotype separately and also with the minor allele AC and AA combined. The complete Kaplan-Meier survival curves were obtained together with log-rank test. All statistical tests were two-sided.

RESULTS

The CCK-B receptor rs1800843 SNP genotype distribution is increased in pancreatic cancer

Our large combined cancer cohort in this current study included DNA from 931 patients with confirmed pancreatic ductal adenocarcinoma. Control subjects were from the central Pennsylvania area or Philadelphia region with comparable ancestry and ethnicity to the cancer cohort. Ancestral genotyping was not performed, but the self-described ancestry for race /ethnicity was reported for 868 of the cancer subjects and included: 4% black, 1%

Asian, and the remaining were Caucasian with at least 6% of this group reported as Hispanics.

The genotype and allelic frequencies in the cohort of pancreatic cancer subjects and controls are shown in Table 1. The frequency of the AA +AC genotypes was greater in the cancer cohort compared to the controls without cancer (p= 0.011). Thirty-seven percent of the pancreatic cancer cohort had one or both A-alleles and this frequency was significantly increased over the 20% occurrence in controls. Compared to our normal cohort, the frequency of the A-allele in pancreatic cancer subjects was also increased (p-value = 0.023; OR = 1.898; 95% CI: 1.0667 3.633). The risk of cancer in those with at least one copy of the A-allele was 2.283 times greater than those with two copies of the C-allele. Thus, those harboring an A- allele of the rs1800843 SNP have an increased risk of pancreatic cancer compared to C allele carriers.

The CCK-B receptor genotype of fifty subjects with histologic diagnosis of pancreatic islet cell or neuroendocrine tumors is shown (Table 1). The allelic distribution for the SNP in the 32^{nd} position of the 4^{th} intron was similar in subjects with islet or neuroendocrine tumors as in those with ductal pancreatic adenocarcinoma; however, the difference compared to controls did not reach significance.

The rs1800843 SNP genotype predicts a phenotype; survival

Accurate survival data or last censored visit date was available in 761 of our patients. Demographics of this cohort are shown in Table 2. There was no significant difference in overall survival across age-at-diagnosis groups (p=0.76) nor between males and females (p=0.99). However, those with more advanced disease at diagnosis (i.e., stage III or IV), as expected, had a shortened survival (Table 2). Therefore, this variable (stage) was further adjusted for in subsequent multivariate analyses.

At the time of diagnosis fewer patients with genotype AA had advanced stage IV disease (28.6%) compared to those with the CC genotype (35.3%); however, this difference did not reach significance (p=0.08). The stage at the time of diagnosis was not statistically different according to genotype. The average stage (mean ± SEM) at the time of diagnosis for each of the genotypes were as follows: AA 2.66± 0.13; AC 2.68 ±0.6; and CC 2.74 ± 0.04). Furthermore, there also was no significant difference between the genotype groups according to the age of diagnosis.

When examining the survival by genotype (AA, AC or CC) significant differences were found ($p= 6.73 \times 10^{-8}$). Independent examination of each genotype revealed that survival was significantly shortened in patients with genotype AC ($p= 7.2 \times 10^{-10}$) and genotype AA ($p= 1.8 \times 10^{-4}$) compared to cancer subjects with the CC genotype (Table 3). Since the AA group was smaller, no significant difference in survival was found between AA and AC subjects. Our prior investigations demonstrated that expression of only one A-allele is necessary to cause the mis-spliced receptor phenotype and extended 3^{rd} intracellular loop if the CCK-BR[28] therefore, a separate combined statistical analysis was also performed for patients with one or two minor alleles. Hence, with combining the AC +AA groups, patients harboring any A-allele in the CCK-B receptor rs1800843 SNP also had significantly shorter

survival (HR =1.56; 95%CI=1.34–1.83; p= 1.8×10^{-8}) by the univariate cox model (Table 3). Survival of those with the C/C genotype was nearly 4 months longer on average compared to those with the A/A genotype. Since stage of disease at diagnosis impacts survival, we performed a multivariate analysis to adjust for stage. Survival was still significantly shortened in those with an A-allele group compared to patients expressing the CC genotype after adjusting for stage of disease (adjusted HR=1.83; 95% CI= 1.53–2.19; p=3.1×10^{-11}) indicating that this germline variant is an important predictor of outcome in pancreatic cancer patients.

Figure 1 shows the survival according to genotype by the Kaplan-Meier analysis. Patients with the A-allele SNP had a more aggressive cancer phenotype with a statistically significant shorter median survival time (MST) compared to patients with the CC wild genotype (9.30 ± 0.73 vs. 11.20 ± 0.74 months, log-rank p= 1.3×10^{-8}). Since over 35% of patients in the pancreatic cancer cohort had an A-allele, this high frequency of expression could contribute to the aggressive nature and poor survival historically reported with this malignancy.

Predicted model for mis-splicing

Using a program that predicts splicing factor binding sites[29,30] we evaluated whether SR-splicing proteins would differentially bind to either the A-allele or the C-allele mRNAs of the CCK-BR. Differences in the predicted SR-protein binding were identified; in particular the SRp55 protein was predicted to have high binding to the C-allele mRNA (Figure 2A) but little or no binding to the A-allele mRNA (Figure 2B). In our proposed model, we suggest that CCK-BR mRNAs that are the wild-type C variant bind the SRp55 SR-protein resulting in normal splicing of the 4th intron. However, in those CCK-BR mRNAs with the A variant SNP, SRp55 does not bind and the 4th intron is not spliced; resulting in the CCK-BR splice variant phenotype (Figure 2C).

DISCUSSION

The present study provides evidence that the frequency of a germline mutation of the CCK-BR is significantly increased in subjects with pancreatic cancer compared to normal controls, suggesting that the mutation could represent a risk for the development of this malignancy. There is also an increased prevalence of the A-allele distribution in pancreatic neuroendocrine tumors, albeit not quite significant. Since neuroendocrine and islet cell tumors of the pancreas have also been shown to over-express CCK-B receptors, this finding may suggest that the A-SNP may somehow be involved with malignant transformation of cells that express the CCK-BR. The other important finding from our investigation is that the presence of the CCK-BR rs1800843 SNP significantly influences the aggressive nature of this disease and shortens survival. Additional evidence that this mutation accelerates growth is suggested by our discovery that even though fewer subjects with the AA genotype presented with advanced (Stage IV) disease compared to the wild-type CC genotype, their survival was still shorter. This finding is supported by the multivariate analysis that confirms that the presence of the variant A-allele alters survival independently after adjusting for stage of disease.

Genetic markers for cancer are identified by one of two major methods: the first is the GWAS that looks for an association between genetic variation and a disease process that differs between control subjects and cancer patients, and the second method is a hypothesis driven candidate-gene association study. Herein, we used the latter method with the underlying hypothesis that mis-splicing of the 4th intron of the CCK-B receptor is the result of a genetic mutation and that this SNP is associated with an aggressive phenotype. This study characterizes the importance of a germline mutation of the human CCK-BR that is significantly related to survival of pancreatic cancer. Since patients with the A-allele have been included in survival studies of pancreatic cancer, it is conceivable that having this germline variant has contributed to the overall reported poor outcome of this malignancy. Furthermore, the mutation occurs in a growth-associated receptor that is over-expressed and ubiquitous in pancreatic cancer. Therapeutic strategies to target this receptor or the splice variant receptor (that results as a consequence of the A-allele SNP), may improve survival of patients with pancreatic cancer.

The frequency of the SNP we identified in the CCK-B receptor is significantly increased in our heterogeneous cohort of sporadic pancreatic cancer patients. Since the rs1800843 SNP is a germline variant, we propose the inclusion of this SNP genotype in analyses using high risk cohorts (i.e., those with a family history of pancreatic cancer or patients with chronic pancreatitis). Although the incidence of family history is unknown in our cohort, we propose that the minor allele frequency may be increased in familial cases of pancreatic cancer. Approximately 10% of those with pancreatic cancer have two or more first degree relatives diagnosed with pancreatic cancer[31] and various mutations have been found in patients with familial pancreatic cancer. One subset of patients with familial pancreatic cancer have been described with mutations in the *BRCA2* gene[32] associated with hereditary breast and ovarian cancer syndromes or alterations in the *CDKN2A* gene[33] associated with a familial melanoma syndrome. Hereditary pancreatitis is an inherited condition associated with mutations in the *PRSS1* gene that also carries a high risk for the development of pancreatic cancer.[31,34] Peutz-Jegher syndrome, another familial disease, is an autosomal dominant disorder in which over 90% of the patients have mutations in the *STK11* gene and an increased risk of developing pancreatic cancer over time.[35] By studying these so-called “familial pancreatic cancer” patients we may gain further insight into the genetics involved with this cancer[34,35] including the frequency of this CCK-BR germline mutation. Since the SNP described in this report is germline and not somatic, DNA screening of patients with a family history of pancreatic cancer could possibly lead to earlier detection and improved outcome.

The first large GWAS studies done for pancreatic cancer utilized exomic sequencing; hence, only areas of genes that were translated into proteins were examined.[36,37] The rs1800843 SNP characterized in our study would not have been identified in whole exome analysis because it is located within an intron. With advanced technology using second generation and deep sequencing, new mutations have been identified including some SNPs within noncoding introns such as a susceptibility locus connected with ABO blood groups[38] and to *NR5A2* that encodes for a nuclear receptor.[39] The CCK-B receptor SNP analyzed in this current project is located on the short arm of chromosome 11p15. Of interest, in a GWAS study of over 1000 pancreatic cancer subjects, Wu and coworkers[40] identified another SNP

(rs10500715) also located on chromosome 11 approximately 4 MB away from the CCK-BR SNP that was also associated with survival in pancreatic cancer patients. It is unknown where there is any relationship between these two SNPs that may occur during DNA folding or cell division. Although several germline variants have been discovered in pancreatic cancer utilizing these more advanced techniques, the clinical significance of many of these SNPs remains uncertain and phenotypic relevance must be validated. The CCK-BR SNP characterized in this report has now been associated with a clinical phenotype, i.e., proliferation and survival.

G-protein-coupled receptors (GPCRs) constitute the largest family of cell surface molecules involved in signal transmission. These receptors mediate key physiological roles and their dysfunction results in several diseases. Many GPCRs are overexpressed in cancers, and contribute to tumor growth when activated by circulating or locally produced ligands.[26] Unlike other solid tumors, G-protein coupled receptors have been shown to play a critical role in mediation of growth of gastrointestinal cancers[26,41] and have been shown to directly interact with more than 15 different proteins that modulate intracellular signaling.[42] GPCRs and their direct effectors (the G-proteins) have been previously described as oncogenes[26] and nearly 20% of human cancers have mutations in GPCRs.[43] In a large pancreatic cancer GWAS analysis, Wei and coworkers[44] screened a database of over 3,000 pancreatic cancer patients and consistently found that the G-protein coupled receptor signaling pathway was the most significant pathway to predict pancreatic cancer risk. Huang et al,[45] found that the AKT signaling protein was significantly over-expressed in pancreatic cancer compared to noncancerous tissues using reverse phase protein array. Indeed, we previously demonstrated that growth of pancreatic cancer is regulated by the over-expression of this GPCR, CCK-BR[21] and that down regulation of the CCK-BR in pancreatic cancer cells results in apoptosis and halts cell proliferation[22] by interference with intracellular signaling and decreased AKT phosphorylation. These investigations support the important role of the GPCR, CCK-BR, in pancreatic cancer.

Our study herein confirms that the addition of amino acid length to the 3rd intracellular loop of the CCK-BR by mis-slicing of the 4th intron contributes to the aggressive nature of pancreatic cancer and shortens survival. This 3rd intracellular loop of the receptor is the part of the receptor that interacts with G proteins and is crucial for intracellular signaling and the resultant induction of cell proliferation. Transfection of wild-type cells with the additional 69-amino acid loop significantly accelerates cell proliferation and can transform cells,[28] and experiments to knockdown expression of the 69 amino acid insertion markedly inhibit cell growth in culture.[46] Moreover, when this insert was stably transfected into HEK293 cells, it induced an interaction with Src kinase[47,48] and increased oncogenesis. The authors proposed that the 69 amino acid insertion rendered the CCK-BR constitutively active[49] increasing its proliferative potential. These reports support the important role of the CCK-BR insert of the splice variant receptor in oncogenesis and cancer growth. Although these past studies have all shown a relationship between the extended splice variant CCK-BR and proliferation of cells *in vitro*, the current report is the first to demonstrate that the splice variant receptor phenotype is clinically relevant and associated with prognosis in human subjects.

As scientists become more sophisticated with their analysis of the human genome, it is important to assure that discoveries of cancer susceptibility loci are validated with phenotypic expression related to the malignancy in question. Researchers have known for years that CCK receptor stimulation induces hyperplasia[50] and inflammation.[51] Since CCK receptors are over-expressed in pancreatic cancer[21] as well as other gastrointestinal malignancies,[52,53] this receptor could become a target for cancer therapy. A better understanding of GPCRs, their role in development and regulation of pancreatic cancer, as well as the rs1800843 SNP genotype may help to improve survival and treatment of pancreatic cancer patients.

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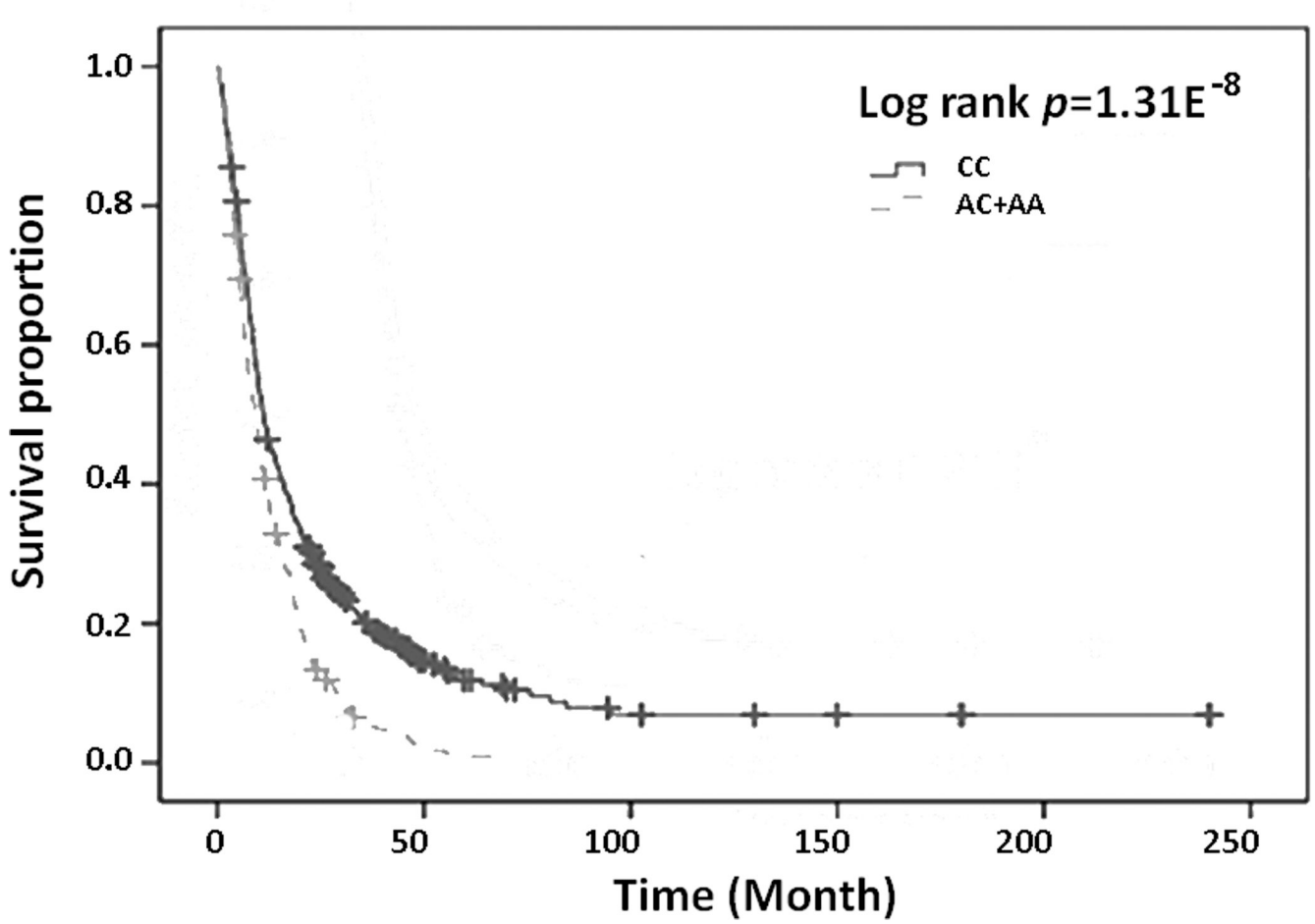

Figure 1.

Survival is shorter in pancreatic cancer patients with an A-allele at the CCK-B receptor rs1800843 SNP. This figure shows the survival according to genotype by the Kaplan Meier analysis. Survival or last censored visit information was available for 761 of the pancreatic cancer patients. Subjects with either one or two A-alleles had a more aggressive cancer phenotype with significantly shortened survival compared to patients with the CC genotype. This difference was significant at a $p = 1.3 \times 10^{-8}$ and a HR of 1.56.

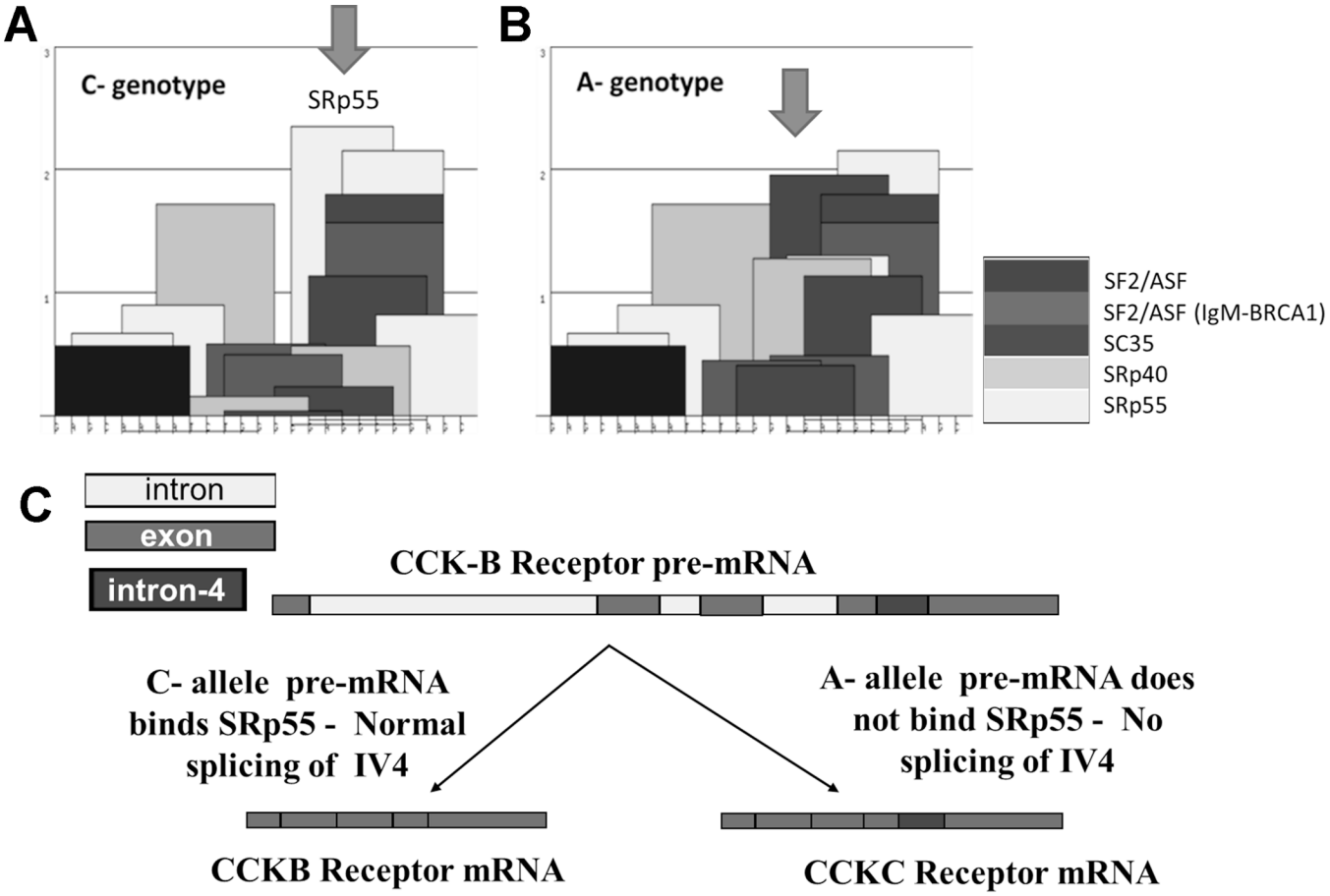

Figure 2.

A model of the CCK-BR SNP genotype splicing interactions. (**A**) Using a splicing factor binding site database several SR-proteins are identified that are involved with splicing intron-4 in the presence of the CC genotype. (**B**) Shows the SR-proteins and absence of the prominent SRp55 protein binding when the A-allele is expressed. The rs1800843 SNP falls within a predicted binding site for the splicing factor SRp55. (**C**) In the presence of the C-allele pre-mRNA binds SRp55 resulting in normal splicing of intron-4 (IV4). In the presence of the A-allele, pre-mRNA does not bind SRp55 and mis-spicing of the 4th intron of the CCK-BR occurs leading to the phenotype of the CCK-C receptor.

Table 1

CCK-BR Genotype (AC+AA) and A-Allelic Frequency is Increased in Pancreatic Cancer Patients Compared to Normal Controls.

Genotype	Pancreatic cancer Cohort N (%)	Normal controls No cancer N (%)	p value PDAC vs. controls	Neuroendocrine Tumors N(%)	p value Neuroendocrine Tumors vs. controls
CC	588 (63.1)	47 (79.7)	0.023**	31 (62)	0.050
AC	307 (33)	10 (16.9)		18 (36)	
AA	36 (3.9)	2 (3.4)		1 (2)	
CC	588 (63.1)	47 (79.7)	0.011**	31 (62)	0.055
AC + AA	343 (36.8)	12 (20.3)		19 (38)	
Allelic frequency (%)	**Pancreatic cancer Cohort**	**Normal controls No cancer**	**p value**	**Neuroendocrine tumor cohort**	**p value**
A-allele	20.4	11.8	0.023**	20	0.1334
C-allele	79.4	88.1		80	

** Significantly different compared to CC genotype. Analysis by the two-sided Fisher's Exact test.

PDAC = pancreatic ductal adenocarcinoma

Table 2

Relationship of Age at Diagnosis, Gender, and Tumor Stage to Survival (n=761)

Characteristic†	No. of cases	No. of deaths (%)	MST± SE (month)	Log-rank *p*	HR (95% CI)
Age at diagnosis (yrs.)				0.7622	
≤50	107	93 (86.9)	10.50 ± 0.80		1.00 (Ref)
51–60	165	143 (86.7)	10.77 ± 0.97		1.00 (0.77–1.29)
61–70	269	241 (89.6)	11.2 ± 0.80		1.02 (0.80–1.29)
>70	220	197 (89.6)	8.5 ± 0.69		1.10 (0.86–1.41)
Sex				0.9933	
Female	311	270 (86.8)	10.00 ± 0.65		1.00 (Ref)
Male	449	403 (89.8)	10.80 ± 0.56		1.00 (0.86–1.17)
Stage				1.62×10^{-30}	
I	48	35 (72.9)	31.03 ± 7.68		1.0 (Ref)
II	271	214 (79.0)	17.50 ±1.37		1.40 (0.98–2.01)
III	72	70 (97.2)	7.70 ± 0.85		3.11 (2.06–4.70)
IV	205	199 (97.1)	6.57 ± 0.58		4.03 (2.79–5.83)

MST: median survival time; SE: standard error; HR: hazard ratio; CI: confidence interval

No differences in survival were found for age at diagnosis or gender, but stage significantly influenced survival.

Table 3

Survival by Genotype Adjusted for Stage of Disease.

***CCKBR* rs1180843**	**No. of cases**	**No. of deaths (%)**	**MST± SE (month)**	**Log-rank *p***	**Crude HR (95% CI)**	**Adjusted HR (95% CI)**	***p* (multivariate)**
Genotypes				6.73×10^{-8}#			
CC	476	397 (83.4)	11.20 ± 0.74		1.00 (Ref)	1.00 (Ref)	
AC	256	249 (97.3)	9.40 ± 0.74		1.54 (1.31–1.81)	1.79 (1.49–2.15)	7.2×10^{-10}*
AA	29	28 (96.6)	7.46 ± 2.27		1.79 (1.22–2.62)	2.30 (1.49–3.56)	1.8×10^{-4}*
Dominant model				1.31×10^{-8}†			
CC	476	397 (83.4)	11.20 ± 0.74		1.00 (Ref)	1.00 (Ref)	
AC+AA	285	277 (97.2)	9.30 ± 0.73		1.56 (1.34–1.83)	1.83 (1.53–2.19)	3.1×10^{-11}*

MST: median survival time; SE: standard error; HR: hazard ratio; CI: confidence interval

*Significant compared to prospective CC group;

#Significant comparing the AA and AC genotypes separated to CC;

†Significant when AA and AC combined are compared to CC (see Kaplan Meier curve Fig. 1).

Adjusted HR = hazard ratio is adjusted for stage of disease.