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A Common CCK-B Receptor Intronic Variant in Pancreatic Adenocarcinoma in a Hungarian Cohort

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Abstract

Objectives—Variant c.811+32C>A in intron 4 of the cholecystokinin-B receptor gene (*CCKBR*) was reported to correlate with higher pancreatic cancer risk and poorer survival. The variant was suggested to induce retention of intron 4, resulting in a new splice form with enhanced receptor activity. Our objective was to validate the c.811+32C>A variant as an emerging biomarker for pancreatic cancer risk and prognosis.

Methods—We genotyped variant c.811+32C>A in 122 pancreatic adenocarcinoma cases and 106 controls by sequencing and examined its association with cancer risk and patient survival. We tested the functional effect of variant c.811+32C>A on pre-mRNA splicing in HEK 293T and Capan-1 cells transfected with *CCKBR* minigenes.

Results—The allele frequency of the variant was similar between patients and controls (18.4% and 17.9%, respectively). Survival analysis showed no significant difference between median survival of patients with the C/C genotype (266 days) and patients with the A/C or A/A genotypes (257 days). *CCKBR* minigenes with or without variant c.811+32C>A exhibited no difference in expression of the intron-retaining splice variant.

Conclusion—These data indicate that variant c.811+32C>A in *CCKBR* does not have a significant impact on pancreatic cancer risk or survival in a Hungarian cohort.

Keywords

pancreatic adenocarcinoma; CCK-B receptor; alternative splicing; survival; genetic risk factor

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has the highest mortality rate of all malignancies. There is no effective screening available and advanced disease is commonly present at initial diagnosis.[1] Established risk factors are cigarette smoking, chronic pancreatitis, diabetes mellitus and increased body mass.[2] Inherited genetic factors also play an important role in familiar and sporadic occurrences of pancreatic cancer. Several highly penetrant susceptibility genes have been identified, including BRCA1, BRCA2, p16/CDKN2A, STK11/LKB, TP53, APC, PRSS1, SPINK1, PALLD and PALB2, which are

mainly associated with familial cancer syndromes and familial pancreatic cancer.[3,4] In sporadic cases more common genetic variants are implicated, that represent a minor risk for the disease, such as variants in the AB0 blood group gene.[5,6] To date, five genome-wide association studies (GWAS) have described multiple susceptibility loci associated with the risk of pancreatic cancer.[5–9] Smith et al. (2012) reported a common single nucleotide polymorphism (SNP) in the cholecystokinin-B receptor gene (*CCKBR*) as a risk factor for PDAC, which has not been observed in prior GWAS studies.[10] The authors showed in a small cohort (51 cases and 39 controls) that variant c.811+32C>A (rs1800843) located in intron 4 of *CCKBR* increased PDAC risk and was also associated with poorer survival. In a more recent follow-up study, Smith et al. (2014) replicated their results in a larger North-American multi-center cohort (931 cases and 59 controls) and confirmed both increased PDAC risk (odds ratio 2.28, CC versus AC plus AA genotypes) and shorter survival (hazard ratio 1.56) associated with variant c.811+32C>A.[11] The gastrin/CCK-B receptor is a member of the G protein-coupled receptor superfamily, physiologically expressed in the human pancreas.[12] Binding of gastrin or CCK triggers activation of multiple signal transduction pathways that relay mitogenic signals to the nucleus and promote cell proliferation. Numerous studies have shown that CCK-B receptor plays a significant role in carcinogenesis and tumor progression.[13,14] An alternatively spliced mRNA form of the receptor generated by retention of intron 4 (designated CCK-BRi4sv for intron 4-containing splice variant; also referred to as CCK-C receptor) was reported in various tumours[15], including pancreatic cancer.[16] The resulting CCK-BRi4sv receptor protein exhibits constitutive (agonist-independent) activation of cell proliferation pathways.[17] Using immunohistochemistry, Smith et al. (2012) found that tumors with variant c.811+32C>A expressed CCK-BRi4sv receptor protein, suggesting that the variant might be directly responsible for intron retention. The authors speculated that binding of the splicing factor SRp55 might be reduced by the intronic variant resulting in enhanced retention of intron 4[10, 11].

There are few known risk factors in pancreatic adenocarcinoma and a better understanding of the molecular pathogenesis is urgently needed. Therefore, we aimed to re-evaluate the role of variant c.811+32C>A as a novel genetic prognostic marker. In this study we had three objectives: (1) to replicate the association between *CCKBR* variant c.811+32C>A and the risk for developing pancreatic cancer in an independent population, (2) to evaluate the impact of the variant on patient survival and (3) to examine the functional effect of the variant on pre-mRNA splicing.

METHODS

Study population

The study protocol has been approved by the Regional and Institutional Committee of Science and Research Ethics. All participants gave written informed consent for genetic analysis. 122 cases with a confirmed diagnosis of PDAC were recruited from the Hungarian National Pancreas Registry. For each patient, information about gender, age at diagnosis, method of diagnosis and date of death or date of last follow-up was collected. Two patients had synchronous or metachronous cancer suggestive of inherited cancer syndromes. Other

cases were sporadic; no patients fulfilled the criteria for familial pancreatic cancer (two or more first degree relatives with pancreatic cancer). 106 control subjects were recruited from adult volunteers who considered themselves generally healthy and from inpatients, who had no history of pancreatic diseases. Characteristics of cases and controls are described in Table 1. and Table 2.

DNA extraction and genotyping

Genomic DNA was isolated from 300 µl EDTA-blood using QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). Primers were designed according to the genomic sequence of *CCKBR* on chromosome 11 (GenBank NC_000011.10) (see primer sequences in Table 3). PCR was performed in a total volume of 30 µl, which contained 0.5 U HotStarTaq DNA Polymerase (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer and 10–50 ng genomic DNA. Amplification was performed under the following cycle conditions: 95 °C for 15 min to activate the enzyme, followed by 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C and 1 min extension at 72 °C, with a final extension of 5 min. Prior to sequencing PCR products were purified with QIAquickPCR Purification Kit (Qiagen). Nucleotide sequence analysis was carried out in a commercial laboratory (Delta Bio 2000 Ltd., Szeged, Hungary) using a 3500 Genetic Analyser (Applied Biosystems) automatic dye-terminator sequencing machine. The reverse PCR primer was used as sequencing primer. Chromatograms were analyzed with ChromasPro software (Technelysium, South Brisbane, Australia).

Construction of expression plasmids harboring *CCKBR* minigenes

We designed *CCKBR* minigenes that contain intron 4 placed in the appropriate context of the full length coding DNA. (Figure 1) *CCKBR* coding DNA (GenBank NM_176875.3) was custom synthesized (GenScript) and cloned into the pcDNA3.1(−) plasmid using XhoI and EcoRI restriction sites. To create *CCKBR* minigenes, a 584 nucleotide long fragment containing intron 4 with or without the c.811+32C>A variant was custom synthesized and cloned into the pcDNA3.1(−) *CCKBR* plasmid using BsrGI and BamHI restriction sites. Full sequences of *CCKBR* minigenes are provided in Supplementary Material.

Construction of lentiviral vectors

The pWPI lentivirus vector plasmid and the packaging plasmids (psPAX2 and pMD2.G) were obtained from Didier Trono's laboratory (http://tronolab.epfl.ch/; Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) through Addgene (Addgene plasmids 12254, 12260, and 12259). First, CCKBR minigene templates were PCR amplified with Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) using the following primers 5'-GCTTAATTAACCATGGAGCTGCTAAAGCTGAACC-3' containing PacI restriction site and 5' phosphorilated 5'-CTCAGCCAGGGCCCAGTGTG-3'. CCKBR minigene inserts were then subcloned into pWPI plasmid between PmeI and PacI restriction sites. The lentivirus production in HEK 293T packaging cells was performed as described previously[18]. Briefly, 293T cells were co-transfected with the pWPI expression plasmids, the packaging plasmid psPAX2 and the envelope vector pMD2.G. Transfection medium was

changed after 16 h, and the lentivirus-containing medium was subsequently harvested after 48 hours and frozen at −80°C. Viral preparations were titrated on HEK 293T cells.

Cell culture, transfection and viral transduction

Human embrionic kidney (HEK) 293T cells were cultured in 6-well plates in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Budapest, Hungary) supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin at 37 °C in a humified atmosphere containing 5% CO_2. Transfections of HEK 293T cells were performed at 70–80% confluence using 2 μg plasmid DNA and 10 μL Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) in 2 ml Opti-MEM Reduced Serum Medium (Life Technologies). After 4 h of incubation, cells were washed and the transfection medium was replaced with 2 ml DMEM. Cells were harvested 24 h after this medium change. Capan-1 pancreatic adenocarcinoma cells were maintained in RPMI-1640 Medium (Sigma) supplemented with 15% fetal bovine serum 4 mM glutamine, and 1% penicillin/streptomycin at 37°C. To establish stable cell-lines a total number of 10^5 cells were plated in 6-well plates and transduced with viral supernatant at multiplicity of infections (MOIs) of four. Expression analysis was performed at first, second and third passage.

RNA extraction and reverse transcription

Total RNA was isolated from transfected cells using RNeasy Mini Kit (Qiagen). To avoid plasmid and genomic DNA contamination, an additional on-column DNase digestion step was applied with RNase-Free DNAse (Qiagen). Two μg RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in the presence of RNase inhibitor RNasin Plus (Promega, Fitchburg, WI, USA).

Quantification of *CCKBR* expression and splicing

Real-time PCR reactions were performed with Maxima SYBR Green/ROX qPCR Master Mix (2×) (Fermentas) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) platform with the following conditions: 10 min initial denaturation at 95 °C, followed by 40 two-step cycles: 15 s at 95 °C and 1 min at 60 °C. Primer sequences are given in Table 3. Threshold cycle (CT) values were determined using the 7000 Sequence Detection System Software V.1.2.3. Relative expression was calculated using the comparative CT method (ΔΔCT method). Expression level of *CCKBR* was first normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) internal control gene (ΔCT) and then to expression levels measured in cells transfected with empty vector (ΔΔCT). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$. Relative expression of splice variants was studied by using two different primer sets, one amplifying both the spliced and unspliced forms of *CCKBR* and the other amplifying the intron 4-retaining splice variant (*CCKB-Ri4sv*) only. For absolute quantification of total *CCKBR* and *CCK-BRi4sv* expression, we generated external calibration curves using serial dilutions of minigene plasmid templates. Using the calibration curves, copy numbers of total *CCKBR* and unspliced *CCK-BRi4sv* were determined and expressed as percent of total (spliced plus unspliced) *CCKBR* expression. All reactions were performed in duplicates.

Statistical analysis

Quantitative variables were described as mean ± SE. Observed genotype frequencies in the study population were compared to the expected Hardy–Weinberg equilibrium. To test the association between pancreatic cancer and genotype/allele frequencies we used two-tailed Fisher's exact test. Additional odds ratios (OR) with 95% confidence interval (CI) were estimated. Overall survival (OS) was defined as the time interval between diagnosis and death (uncensored observation) or the last date when the patient was still known to be alive (censored observation). Survival curves were calculated for OS of patients according to Kaplan-Meier. Two-sided log rank test was used to compare the difference between survival of pancreatic cancer patients harboring the A-allele (A/A or A/C) with survival of those patients with the C/C genotype. Median survival time was calculated using data from all patients; median follow-up time was computed with censored observations. All the analyses were performed with GraphPad Prism (San Diego, CA, USA). For sample size calculation we used Quanto v.1.2.4.[19].

RESULTS

Variant c.811+32C>A does not predict risk for PDAC

First, we attempted to replicate the published association between variant c.811+32C>A in intron 4 of *CCKBR* and the risk for developing pancreatic cancer. To this end, we sequenced this region of *CCKBR* in our Hungarian cohort and detected variant c.811+32C>A in 35 heterozygous and 5 homozygous cases (allele frequency 18.4%), and in 32 heterozygous and 3 homozygous controls (allele frequency 17.9%). Similarly to allele frequencies, genotype frequencies did not show a statistically significant difference between cases and controls either (Table 4). Genotype frequencies in cases and controls were found to conform to the Hardy-Weinberg equilibrium. Additionally, we identified two variants in exon 5: c.955C>T (p.R319W, rs113168010) in one control subject and c.956G>A (p.R319Q, rs1805001) in a single patient (Figure 2).

Variant c.811+32C>A does not predict survival in PDAC

To address the hypothesis that variant c.811+32C>A may have prognostic relevance, we analyzed this variant in relation to patient survival (Figure 3). Median follow-up time was 334 days, 12.3% of the observations were censored. Median survival of cases with A/C and A/A genotypes was not significantly different from those with the CC genotype (257 days and 266 days, respectively; p=0.45), indicating that this variant does not modify survival of PDAC patients.

Variant c.811+32C>A does not affect splicing of intron 4 in *CCKBR*

To determine whether variant c.811+32C>A has an effect on pre-mRNA splicing; we tested two different cell lines. HEK 293T cells were transfected with expression plasmids carrying *CCKBR* minigenes with or without the intron 4 variant and examined mRNA expression. *CCKBR* expression in transfected HEK 293T cells was about six orders of magnitude higher than endogenously expressed levels (Figure 4A). Interestingly, *CCKBR* mRNA was expressed at 1.5-fold higher levels when cells were transfected with intron-containing

minigenes compared to cells transfected with the intronless *CCKBR* cDNA construct. This phenomenon is in agreement with published observations that the presence of introns can enhance gene expression.[20,21] For absolute quantification of different splice-forms, we generated calibration curves using minigene plasmids as template. We found that expression of the *CCK-BRi4sv* intron 4-retaining splice variant corresponded to about 10% of total *CCKBR* mRNA and was not different between cells transfected with minigenes with or without the c.811+32C>A variant (Figure 4C). Since splicing factors can be differently expressed in carcinoma cells, we have analyzed *CCKBR* splicing in Capan-1 pancreatic adenocarcinoma cells as well. In line with the data on HEK 293T cells, splicing was not affected by the variant c.811+32C>A (Figure 4B, 4D).

DISCUSSION

Identification of pancreatic cancer susceptibility genes is of outmost importance to define high-risk populations who may benefit from early detection by screening tests. Based on its role in pancreatic carcinogenesis and regulation of tumor growth *CCKBR* is a promising candidate for a susceptibility gene. Indeed, several somatic mutations were identified in colorectal and gastric cancers that alter receptor activity, sensitization and localization.[22,23] Some of these mutations are located in the third intracellular loop of the receptor, which plays a critical role in signal transduction. The same loop is altered by the tumor associated *CCK-BRi4sv* splice variant, which retains intron 4 and codes for an insertion of 69 additional amino acid residues that enhances receptor activity.[17] The molecular basis for this alternative splicing has been explained by aberrant expression of certain auxiliary splicing factors in carcinoma cells that are necessary for the spliceosome assembly.[24] Alternatively, Smith et al. (2012, 2015) proposed that the c.811+32C>A intronic variant in *CCKBR* can induce retention of intron 4 and thereby increase risk for the development of PDAC and also lead to poorer survival in carriers [10, 11]. In contrast, here we demonstrated that variant c.811+32C>A has no effect on *CCKBR* mRNA splicing, and it is not associated with increased risk for pancreatic cancer, nor with shorter survival in PDAC. Although we had more than 85% statistical power to replicate the previously described odds ratio of 2.28, we detected no enrichment of the variant in our PDAC cohort. The reasons for the discrepancy between our results and those of Smith et al. (2012, 2015) are not readily apparent but may be related to ethnic and geographic variability of the frequency of the c.811+32C>A variant and the admixed nature of the US cohort. Association studies in ethnically admixed populations are potentially vulnerable to spurious association due to the ethnic variability of the SNP frequency studied. Indeed, data retrieved from the 1000 Genome Project database (www.1000genomes.org) show that the allele frequency of variant c.811+32C>A is 18.4% in subjects of European origin, whereas it is 2% in subjects of Asian descent and 23% in subjects of African descent. We also note that the control group in the study by Smith et al. (2015) was unusually small (59 subjects), which might result in the incorrect determination of control genotype frequencies. Indeed, the reported minor allele frequency (11.8%) for this control cohort is appreciably smaller than the incidence found in our controls (17.9%) which compares well with the 1000 Genomes data.

In conclusion, data presented here argue that intronic variant c.811+32C>A in *CCKBR* is not associated with PDAC risk or survival in a Hungarian cohort and does not alter splicing of

the *CCKBR* pre-mRNA. Despite the fact that our study was not designed to detect a potentially small effect of variant c.811+32C>A on cancer risk and we did not take into account age and tumor stage at diagnosis when analyzing survival, our findings are convincingly self-consistent. Therefore, we propose that variant c.811+32C>A is functionally harmless and it should be considered a common polymorphism with no clinical significance. Finally, our results highlight the necessity for replication studies and the importance of functional testing of new genetic risk markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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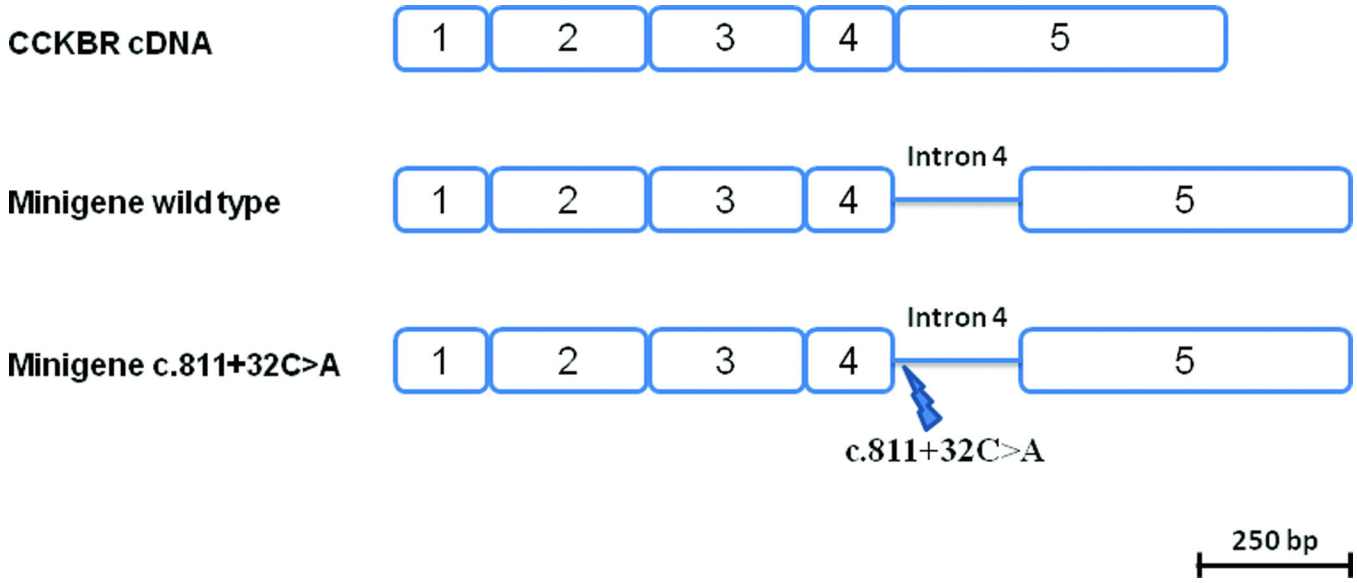


Figure 1.

Minigene constructs used to analyze the effect of the c.811+32C>A variant on pre-mRNA splicing. Intron 4 was placed in the appropriate context of the *CCKBR* cDNA. Numbers indicate exons.

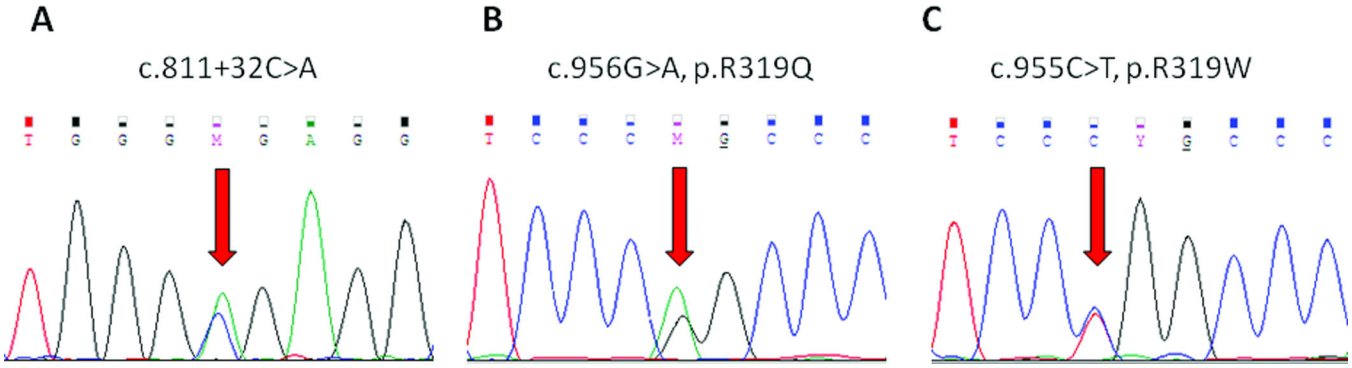


Figure 2.
Sequence electropherograms of *CCKBR* gene variants found in our cohort.

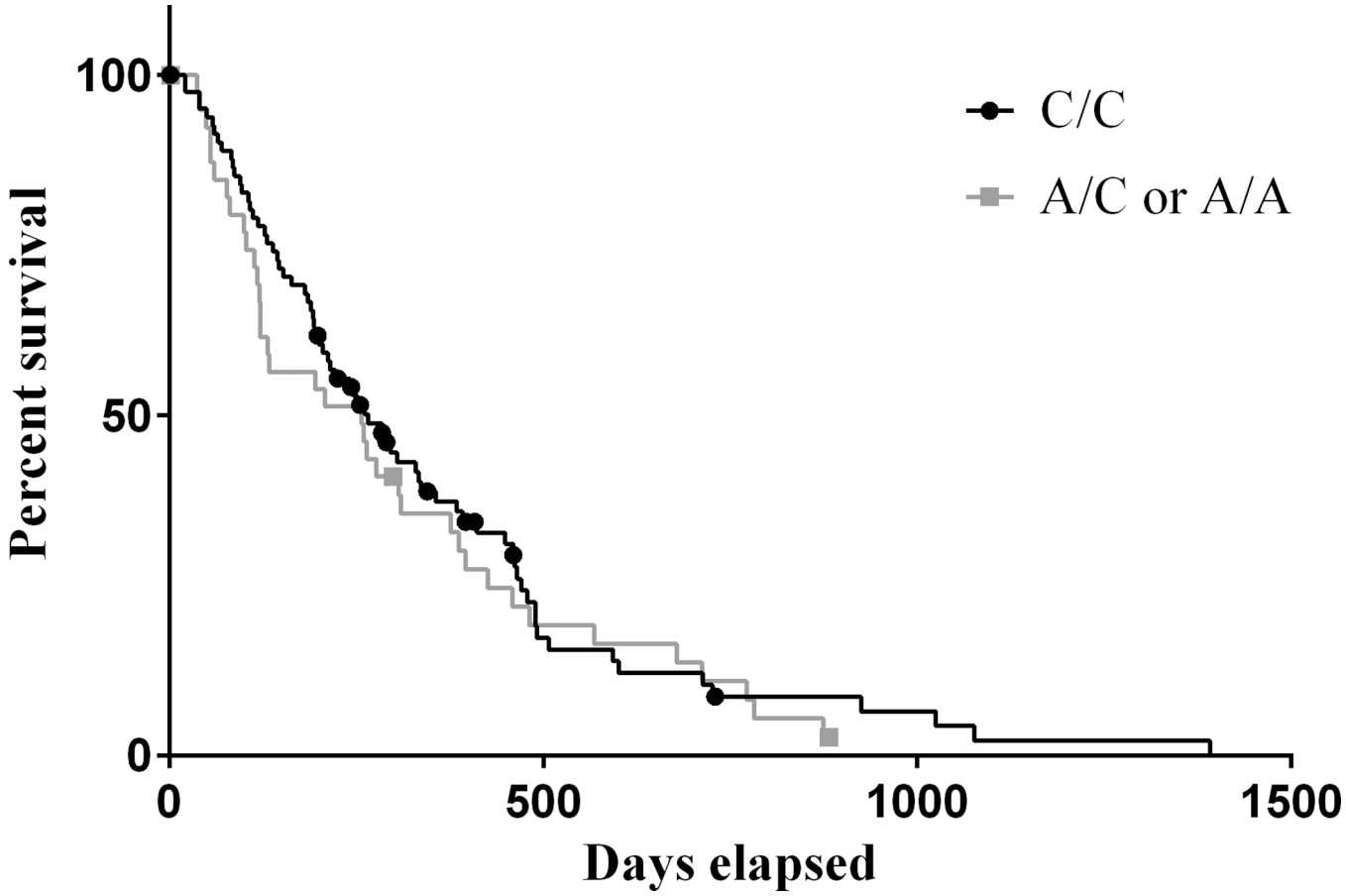
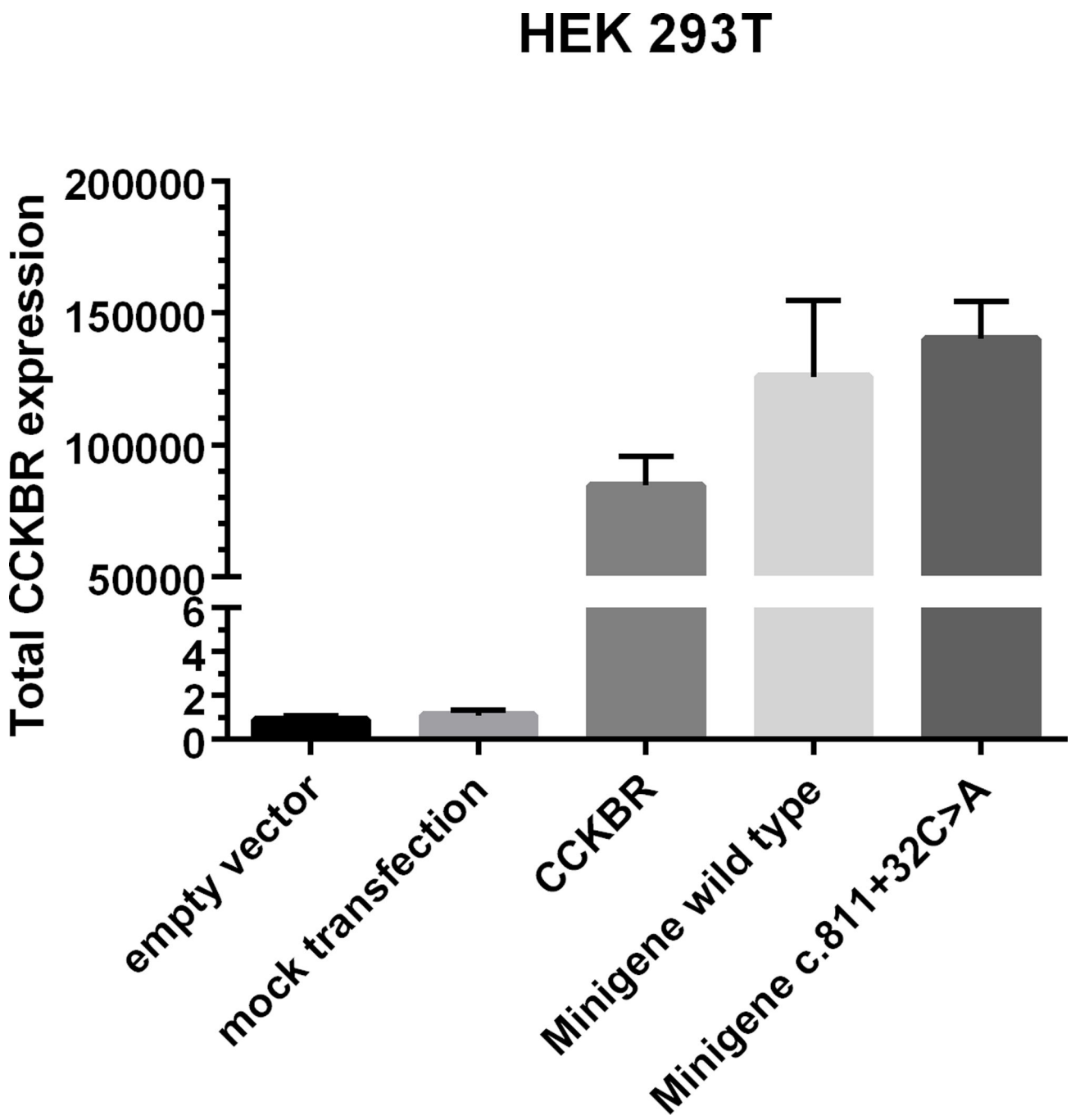
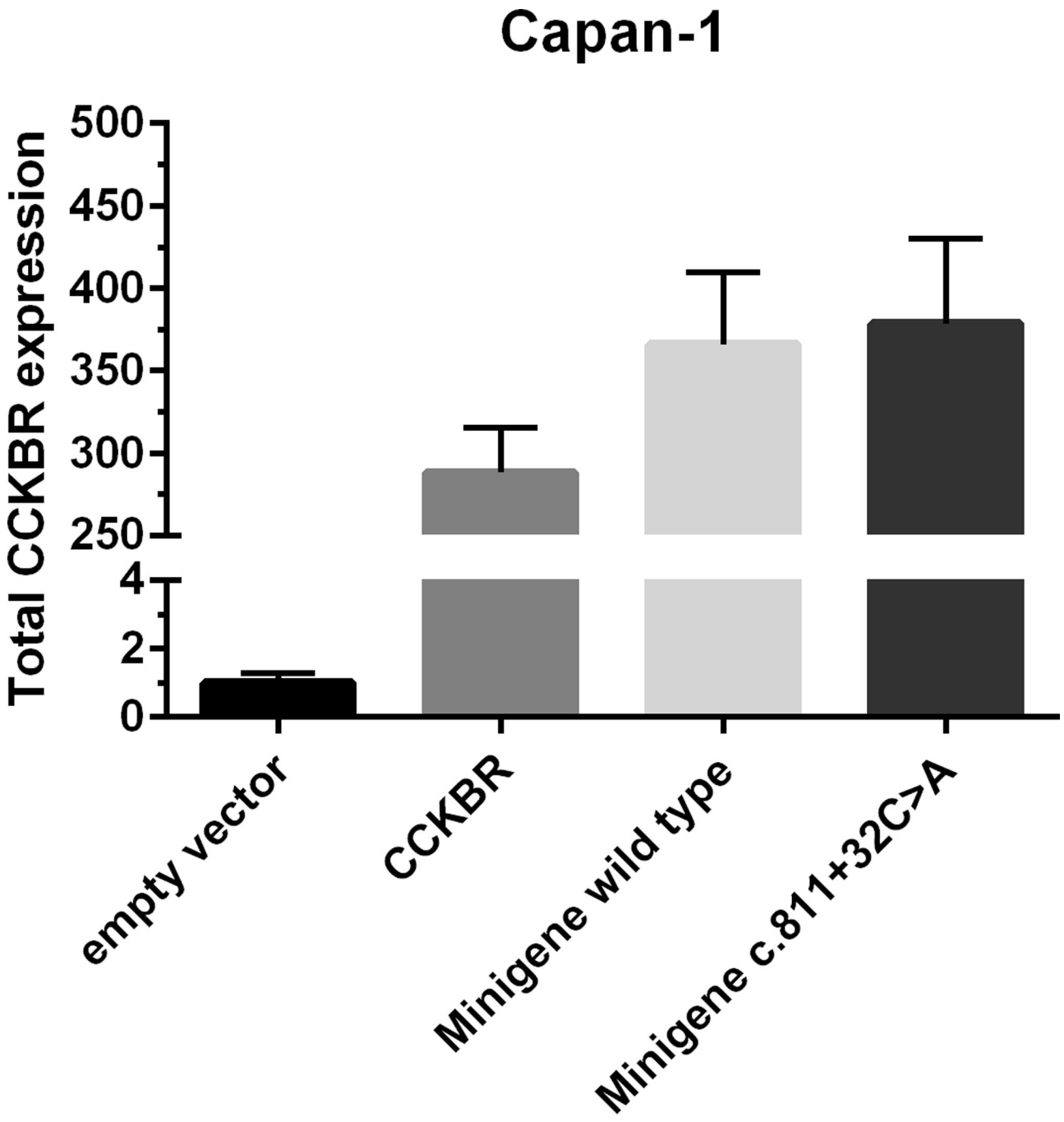
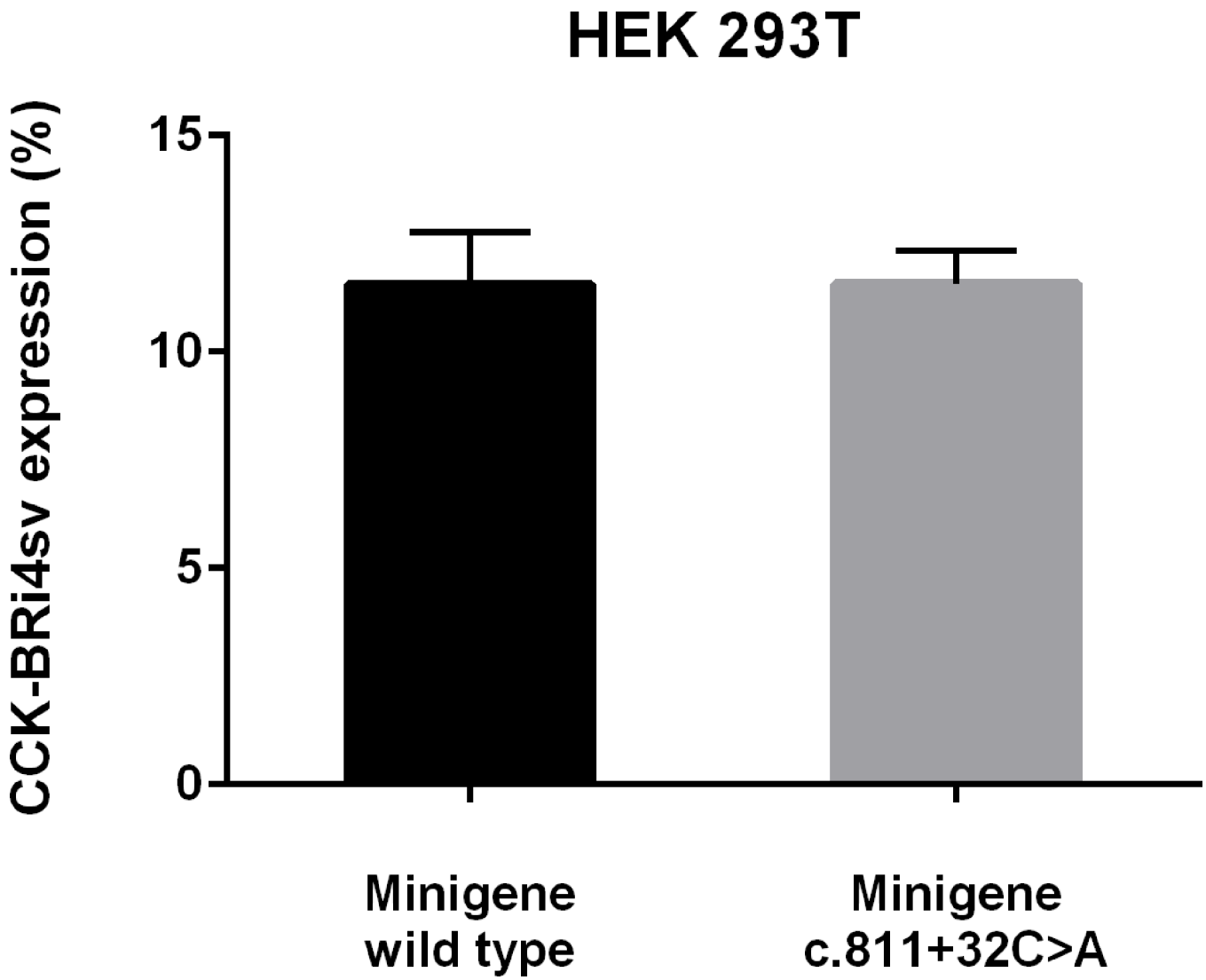


Figure 3.
Kaplan-Meier survival curves according to genotype. Censored cases are shown as dots and squares.







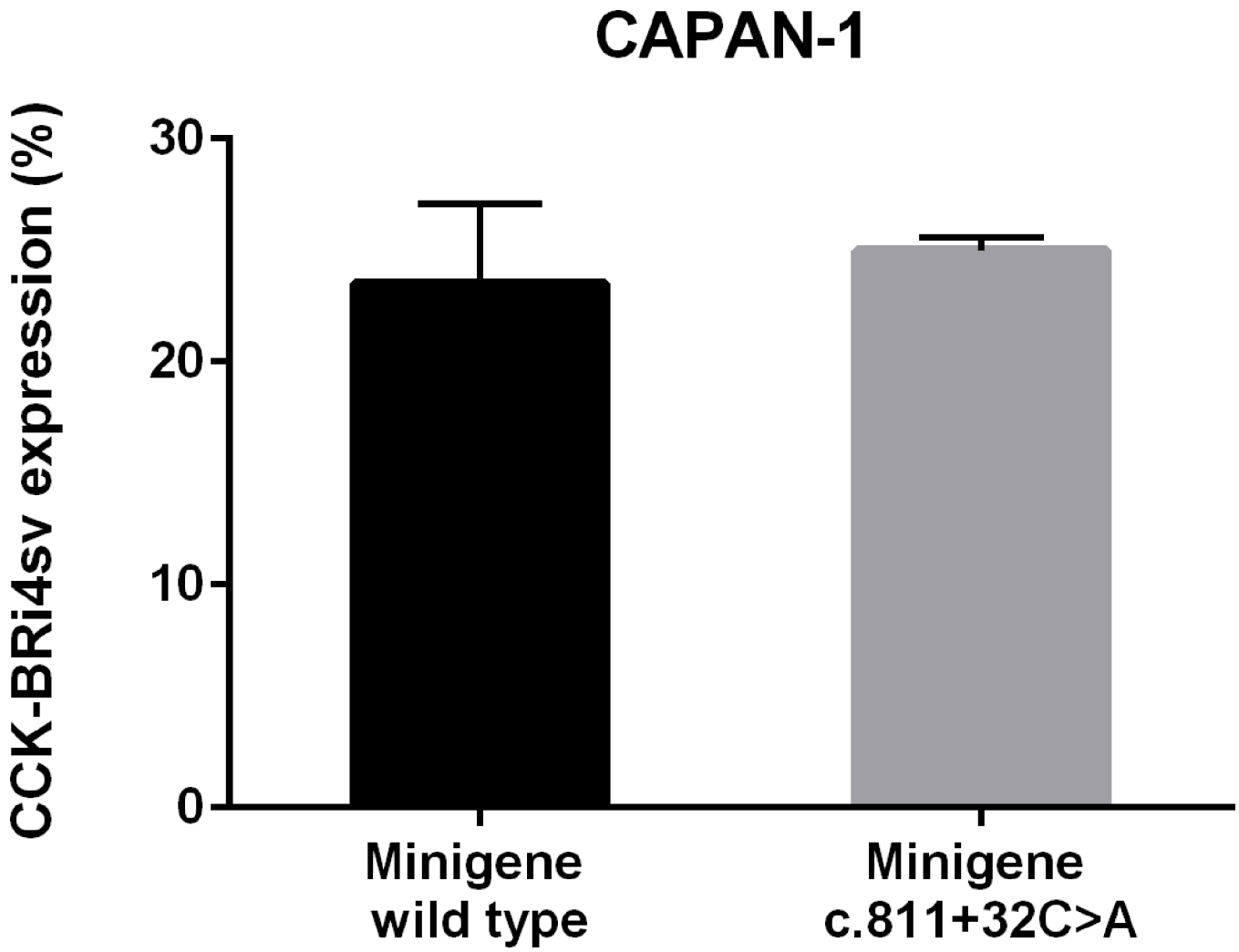


Figure 4.

Functional analysis of the effect of variant c.811+32C>A on pre-mRNA splicing. Expression of *CCKBR* mRNA in transfected HEK 293T cells (**A**) and transduced Capan-1 cells (**B**) with the indicated constructs. Expression of the intron-retaining splice variant relative to the total amount of *CCKBR* mRNA in HEK 293T cells (**C**) and Capan-1 cells (**D**).

Table 1

Characteristics of patients and controls. PDAC, pancreatic ductal adenocarcinoma.

	PDAC Cases	Controls
N	122	106
Gender		
Female	59	61
Male	63	45
Age at diagnosis/recruitment		
Mean±SD	65.6±10.4	51.5±18.8
Age range	31–89	18–89
Survival, days		
Mean±SD	336±251.8	
Median (25%–75%)	260 (118–465)	

Table 2

Tumor stage and survival of patients

	no. of cases	**median survival±SD, days**
localized	9	480±312
locally advanced	79	321±267
metastasized	29	222±204
unknown	5	

Table 3

Oligonucleotide primers used in this study.

Primers used for genotyping		
	forward	*5'-CTGTGTTGCCTTCAGGTCCG-3'*
	reverse	*5'-ATCACCAGCAACATTCGCAC-3'*
Primers used for RT-PCR		
CCKBR-total	forward	*5'-TCTCCTCAACAGCAGCAGTG-3'*
	reverse	*5'-CCCAGGACCACGATGATGAG-3'*
CCKB-Ri4sv	forward	*5'-AATGGAGTTGAGCTGGGAGC-3'*
	reverse	*5'-TGGGCGGTCAGAGAAAAAGG-3'*
GAPDH	forward	*5'-CACCATCTTCCAGGAGCGAG-3'*
	reverse	*5'- GACTCCACGACGTACTCAGC -3'*

Table 4

Genotype and allele frequencies of variant c.811+32C>A in PDAC patients and controls. OR, odds ratio; CI, confidence interval.

	PDAC cases	Controls	genotypic OR (95% CI)	p value
CC	82/122	71/106	Reference	-
AC	35/122	32/106	0.947 (0.5328–1.683)	0.884
AA	5/122	3/106	1.443 (0.339–6.255)	0.7271
AC+AA	40/122	35/106	0.9895 (0.5686–1.722)	1
			allelic OR (95% CI)	
Minor allele frequency	18.4%	17.9%	1.01 (0.58–1.76)	1