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Association of a Genetic Variant in the Cyclin-Dependent Kinase Inhibitor 2B with Risk of Pancreatic Cancer

Newsha Sardarzadeh[#1], Fatemeh Khojasteh-Leylakoohi[#2,3], Sedigheh Damavandi[#2], Ghazaleh Khalili-Tanha[3,4], Mohammad Dashtiahangar[2], Nima Khalili-Tanha[2], Amir Avan*[2,3,4,5] Sakineh Amoueian[6], Seyed Mahdi Hassanian[2,3], Habibollah Esmaily[2,3], Majid Khazaei[2,3], Gordon Ferns[7], Alireza Khooei*[6], *Mohsen Aliakbarian[1]

Abstract

Background: Pancreatic cancer (PC) is among the most aggressive tumors with a poor prognosis, indicating the need for the identification of a novel prognostic biomarker for risk stratifications. Recent genome-wide association studies have demonstrated common genetic variants in a region on chromosome 9p21 associated with an increased risk of different malignancies.

Methods: In the present study, we explore the possible relationship between genetic variant, rs10811661, and gene expression of CDKN2B in 75 pancreatic cancer patients, and 188 healthy individuals. DNAs were extracted and genotyping and gene expression were performed by TaqMan real-time PCR and RT-PCR, respectively. Logistic regression was used to assess the association between risk and genotypes, while the significant prognostic variables in the univariate analysis were included in multivariate analyses.

Results: The patients with PDAC had a higher frequency of a TT genotype for rs10811661 than the control group. Also, PDAC patients with dominant genetic model, (TT + TC), was associated with increased risk of developing PDAC (OR= 14.71, 95% CI [1.96-110.35], p= 0.009). Moreover, patients with CC genotype had a higher expression of CDKN2B, in comparison with TT genotype.

Conclusions: Our findings demonstrated that CDKN2A/B was associated with the risk of developing PDAC, supporting further investigations in the larger and multicenter setting to validate the potential value of this gene as an emerging marker for PDAC.

Keywords: CDKN2A/B, Rs10811661, Pancreatic cancer, Prognostic biomarker.

Introduction

Pancreatic cancer is among the aggressive cancer with a poor prognosis, its 5-year survival rate is about 3-15% (1, 2). The most common malignant neoplasm of the pancreas is pancreatic ductal adenocarcinoma (PDAC), which is usually used interchangeably (3). The poor prognosis of PDAC is due to a number of variables, including a lack of appropriate

1: Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.
2: Metabolic syndrome Research center, Mashhad University of Medical Sciences, Mashhad, Iran.
3: Basic Medical Sciences Institute, Mashhad University of Medical Sciences, Mashhad, Iran.
4: Medical Genetics Research center, Mashhad University of Medical Sciences, Mashhad, Iran.
5: Department of Medical Genetics and Molecular Medicine, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
6: Department of Pathology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
7: Brighton & Sussex Medical School, Division of Medical Education, Falmer, Brighton, Sussex BN1 9PH, UK.
***Corresponding authors: Alireza Khooei; Tel: +98 9155154082; Email: KhooeiAR@mums.ac.ir & Mohsen Aliakbarian; Tel: +98 9155111367; Email: AliakbarianM@mums.ac.ir.**
#These authors equally contributed to this study as the first authors.
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markers for early detection, rapid progression, and the paucity of effective treatment options. Early detection of pancreatic cancer patients is one of the most substantial challenges which can meaningfully lower mortality and increase surveillance (4, 5). Several factors have a role in the various types of pancreatic cancer, while tumor suppressors and oncogenes mutations are the most prevalent. Germline mutations in genes such as BRCA1, BRCA2, STK11/LKB, p16/CDKN2A, PALB2, ATM, and DNA mismatch repair genes have been linked to pancreatic cancers (6). However, frequencies of these mutations do not match with the bulk of genetic susceptibility to pancreatic cancer. The cyclin-dependent kinase (CDK) inhibitors CDKN2A (ENSG00000147889) and CDKN2B (ENSG00000147883) exhibited essential role in various disorders. The CDKN2A gene is located on the human genome's chromosome 9 (p21.3) (7). Research has linked CDKN2A/B rs10811661 to an increased risk of numerous malignancies, including breast cancer, esophageal squamous cell carcinoma, and colorectal cancer (8-12). The CDKN2A gene encodes two proteins: INK4 family member p16 or p16INK4a and ARF tumor suppressor or p14arf (13). CDKN2A activity prevents cancer and may speed up the aging process. Uncontrolled cell proliferation caused by the loss of CDKN2A activity due to inhibition, deletion, mutation, or promoter methylation has been observed in a variety of malignancies.

An increased risk of pancreatic cancer has been discovered in several families with inherited melanoma syndromes. Pathogenic mutations in the CDKN2A gene increase the lifetime risk of cutaneous melanoma by 70% and pancreatic ductal adenocarcinoma by 20% (14-16). Carriers might be enrolled in a pancreatic cancer surveillance program because of their elevated PDAC risk (17). Ghiorzo et al. by analyzing CDKN2A mutations in 225 Italian patients with pancreatic cancer, reported that a significant subset of familial pancreatic cancer families may carry CDKN2A mutations (18). Campa et al. examined 13 polymorphisms of the CDKN2A gene and found that the A allele of rs3217992 SNP has the strongest association with an increased risk of PDAC (6). Recently, Overbeek et al. revealed that pathogenic CDKN2A mutations impacting the p16INK4a protein, including c.67G>C, are associated with a higher PDAC risk (15). Regarding theoretical evidence and lack of studies, we aimed to discover the relationship between CDKN2A/B- rs10811661 SNP and PDAC and evaluate the prognostic role of the rs10811661 SNP in PDAC.

Materials and Methods

Patients

In this study, 75 pancreatic cancer patients and 188 age-matched healthy controls from Imam Reza and Sina hospitals of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1400.709). The guidelines of the Mashhad University of Medical Sciences Ethics Committee were used to obtain Informed consent from all the participants. Patient samples were obtained with a confirmed histological diagnosis and with locally advanced or metastatic PC during May/2006-August/2020. The healthy control group was obtained as part of the Mashhad Stroke and Heart Atherosclerotic Disorders (MASHAD) cohort study (27). These individuals had no family history of stroke, cancer, myocardial infarction, infectious disease, and diabetes mellitus.

Genotyping

The genomic DNA was extracted from samples according to the manufacturer's instructions. The NanoDrop®1000Detector (NanoDropTechnologies, Wilmington, DE) was used to determine the concentration and purity of DNAs. Genotype analysis of CDKN2A/B-rs10811661 polymorphism was carried out using the Taqman-® probes-based assay; polymerase chain reactions were carried out in a total volume of 12.5 μL, using 10 ng of DNA in the TaqMan® Universal Master Mix with specific primers and probes (C90179210 and C79005710; Applied Biosystems, Foster City, CA). The allelic content of the samples

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was assessed using an ABIPRISM-7500 analyzer with SDS version 2.0 software.

RT-PCR

RNA was isolated from PDACs using total RNA extraction kit according to the manufacturer's instructions (Parstous, Tehran, Iran). RNA was evaluated by a Nanodrop 2000 spectrophotometer (BioTek, USA EPOCH). cDNA was synthesized using a cDNA synthesis kit (Parstous, Tehran, Iran). Quantitative real-time PCR was performed using specific primer for CDKN2A/B (Macrogene Co., Seoul, South Korea). Quantitative real-time PCR was performed using the SYBR green master mix (Parstous Co. Tehran, Iran), and performed by ABI-PRISM StepOne instrument (Applied Biosystems, Foster City, CA). Gene expression data were normalized to GAPDH, using a standard curve of cDNAs purchased from Quantitative PCR Human Reference RNA (Stratagene, La Jolla, CA) (18).

Statistical analysis

We used SPSS-20 software (SPSS Inc., Chicago, IL) for statistical analysis. The mean and standard deviations were utilized for continuous variables, whereas frequencies and percentages were used for categorical variables in the descriptive statistics of patients with PDAC. The Pearson χ distribution was used to measure deviation from the Hardy–Weinberg equilibrium (HWE) of the CDKN2A/B rs10811661 polymorphism genotype and allele frequencies. Logistic regression was used to analyze the relationship between PDAC risk and genotypes in dominant, recessive, and co-dominant genetic models**.** The odds ratio and its related 95 % confidence interval were used to show PDAC risk estimations**.** For categorical variables, Pearson's chi-square test was used, and Student's t tests were used to analyze the connection between CDKN2A/B rs10811661 polymorphism and clinical pathological characteristics. The statistical significance level of p was 0.05.

Results

Clinicopathological characteristics of patients

Sixty-nine patients with pancreatic cancer were enrolled in the current study. The control group consists of 148 age and sex-matched subjects. Table 1 shows the demographic, clinical, and genetic features of the population. The case group consists of 35 (50.7%) males and 34 (49.3) females, with a mean age of 61.46 ± 11.69 years. According to tumor size, no patients were categorized in the T1 stage (cancer cells invaded the submucosa). In 51.5% of patients, cancer cells invaded the muscularis propria (T2), in 38.2% of cases, cancer cells invaded the adventitia (T3), and the tumor depth in 10.3% of participants was at T4 status, meaning cancer cells invaded contiguous structures. Moreover, in 62% of patients, the cancer progressed to one or two adjacent lymph nodes (N1), whereas distant lymph nodes or other tissue progression was seen in 13% of patients (M1) (Table 1).

Genetic variant and PDAC

Allele and genotype frequencies of rs10811661 polymorphisms are shown in table 2, which was consistent with the HWE (p= 0.09). For rs10811661, minor allele frequencies for T and C alleles were 0.34. In the PDAC group, the frequencies of TT, TC, and CC genotypes for rs10811661 were 75.4%, 23.2%, and 1.4%, respectively, while in the control group, they were 33%, 47.4%, and 19.6%, respectively (Table 2). Hence, we assumed the T allele as a risk allele for further analysis. Under three different genetic models, binary logistic regression analysis of the rs10811661 polymorphism and pancreatic cancer demonstrated a link between PDAC and the rs10811661 polymorphism (Table 3). Using dominant genetic model, the genetic variant (TT + TC) was found to be highly associated with an increased risk of developing PDAC (OR= 14.71, 95% CI [1.96-110.35], p= 0.009). Also, cases with CC genotype were associated with lower expression of CDKN2A/B (Fig. 1).

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Table 1. Clinicopathological characteristics of the study groups, means±SD or N (%).

Characteristic	Patients (%)
Age	61.46±11.69
Sex	
Female	34 (49.3%)
Male	35 (50.7%)
TNM classification	
Stage I-II	36 (52%)
Stage III-IV	33 (48%)
Tumor size	
T1	0 (0%)
T2	35 (51.5%)
T3	26 (38.2%)
T4	7 (10.3%)
Nodal status (N1)	
Yes	43 (62%)
No	26 (38%)
Distant metastasis (M1)	
Yes	9 (13%)
No	60 (87%)
Grade	
Poor-differentiated	0 (0%)
Moderated-differentiated	3 (4.4%)
Well-differentiated	8 (11.6%)
Undifferentiated	58 (84%)

Table 2. Allele and genotype frequencies of rs10811661 polymorphisms.

Gene	SNP	Major/minor allele	Major allele homozygote (%)	Heterozygote (%)	Minor allele homozygote (%)	MAF	HWE (p-Value)
CDKN2A	rs10811661	T/C Control (n= 148)	101 (46.5%) Case (n= 69)	86 (39.6%) Total (n= 217)	30 (13.8%)	0.34	0.09
	TT	49 (33%)	52 (75.4%)	101 (46.5%)			
	TC	70 (47.4%)	16 (23.2)	86 (39.6%)			
	CC	29 (19.6%)	1 (1.4)	30 (13.8%)			

Abbreviations: HWE: Hardy Wienberg equilibrium; MAF: minor allele frequency; SNP: single-nucleotide polymorphism.

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Table 3. Binary logistic regression analysis of rs10811661 polymorphism and pancreatic cancer under different genetic models.

Models	Genotype	Case n (%)	Control n (%)	OR (95%CI)	p-value
Allele	T C	120 (87%) 18 (13%)	168 (56.7%) 128 (43.3%)		
Dominant	TT + TC CC	68 (98.5%) 1 (1.5%)	119 (80.4%) 29 (19.6%)	14.71 (1.96-110.35) 1.00 (Reference)	0.009
Recessive	TC + CC TT	17 (24.6%) 52 (75.4%)	99 (67%) 49 (33%)	1.00 (Reference) 4.65 (2.48-8.75)	0.000
Co-dominant	TT + CC TC	53 (76.8%) 16 (23.2%)	78 (52.7%) 70 (47.3%)	1.00 (Reference) 2.49 (1.31-4.75)	0.005

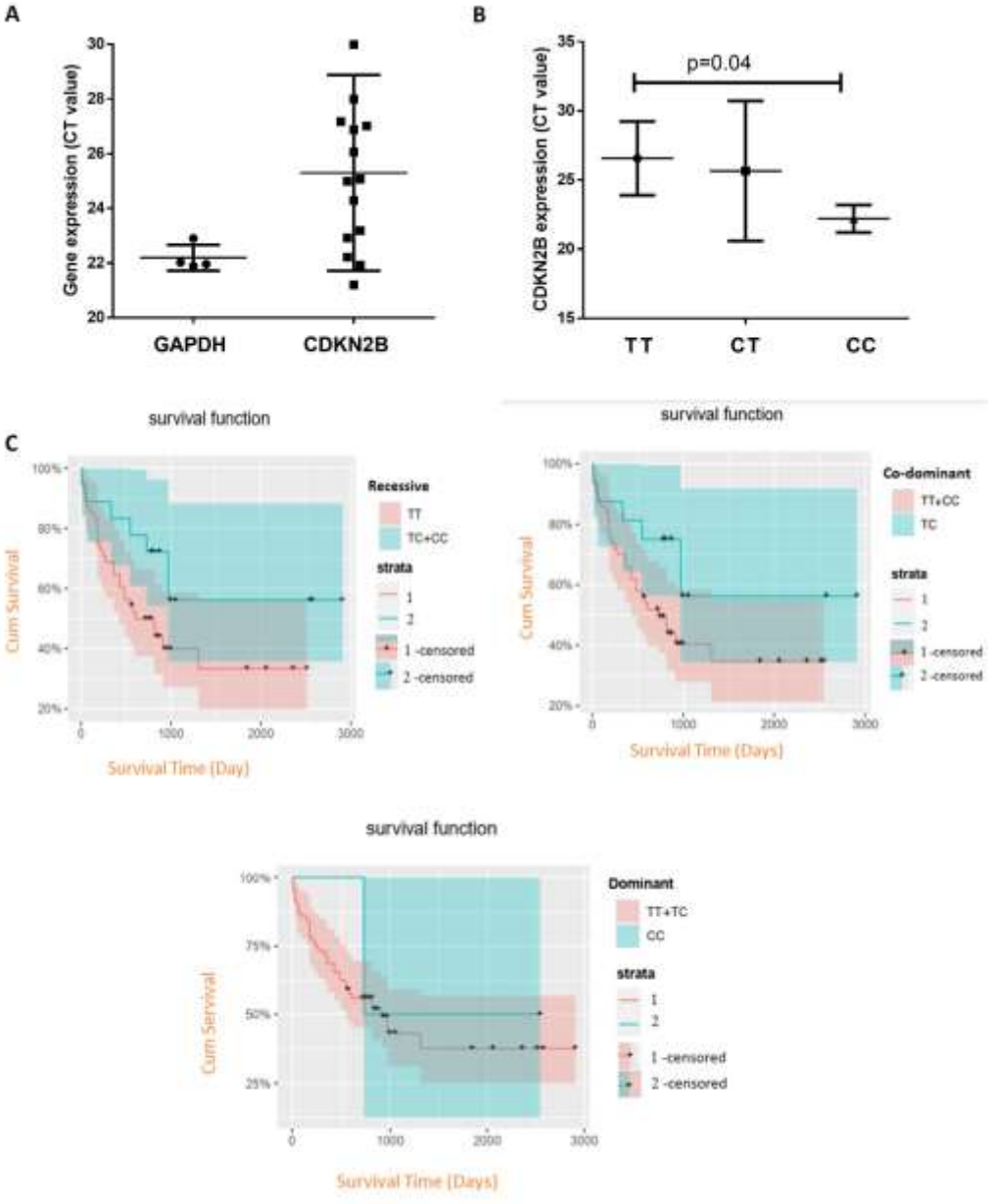

Fig. 1. A-B) CDKN2A/B gene expression in PDAC cases and (C) survival analysis in different genetic models.

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Discussion

Our data illustrated that CDKN2A/B was related with risk of developing PDAC, which is in line with previous observations (15, 19, 20). Previous studies identified a number of CDKN2A/B genetic variations correlated with the risk of multiple cancers. They hypothesized that this region is involved in various types of cancer. The mechanism of this gene's contribution to the cell cycle helps explain the probable link between its actions and PDAC. Genetic variants in the region have also been linked to type 2 diabetes mellitus (T2DM), which is a risk factor for pancreatic cancer, implying that the 9p21.3 region may play a role as a genetic link between the two diseases (6). Previous studies reported a positive correlation between CDKN2B-rs10811661 and impaired pancreatic beta cell function and diabetes (21-23). Overexpression of CDKN2A/2B has been found in clinical and experimental investigations to inhibit pancreatic island proliferation and lead to diabetes, whereas its suppression leads to cell proliferation and cancer (24).

The CDKN2A gene produces two proteins: p16INK4a, a member of the INK4 family, and p14arf, a tumor suppressor. P16 has been found in a variety of tissues, including the pancreas**.** p16INK4a mutations appear to be a primary source of Rb pathway dysfunction in a range of human cancers. The p16INK4a tumour suppressor gene produces a cyclin-dependent kinase inhibitor that binds to CDK4 and CDK6, preventing them from coupling with d-type cyclins and thereby initiating Rb phosphorylation. As a result, it's thought that p16INK4a's dysfunction leads to uncontrolled cell cycle progression and neoplastic transformation (25). p14ARF operates as a tumor suppressor by neutralizing MDM2-mediated p53 degradation (26-28).

Furthermore, inactivation of CDKN2A by methylation or ANRIL can inhibit the activity of tumor suppressor genes. ANRIL controls cell proliferation and senescence indirectly by three methylations of lysine 27 in chromosome 9P21 (3meH3K27). ANRIL is a 3.8 kb lncRNA that is transcribed in the opposite way as the p15/CDKN2Bp16/CDKN2Ap14/ARF gene cluster and has been linked to the recruitment of EZH2 (PRC2) and CBX7 (PRC1) complexes to certain loci (29-31). The rs10811661 SNP affects ANRIL expression, which can influence CDKN2A expression and lead to cell hyper-proliferation (32).

Previously, Campa et al. showed that among 13 SNPs in the 9p21.3 region, only rs3217992 was significantly associated with an increased risk of developing PDAC (6). The rs3217992 SNP is located in a miRNA target region (miR-138-2-3p). It may affect CDKN2B by interfering with miRNA-mRNA interactions (33). A recent meta-analysis of 45 case-control studies concluded that COX-2-765, HIF-1 rs11549467, and TP53 rs9895829 additive genetic models, as well as dominant gene models of DR rs2228570, CTLA-4 rs231775, and MTHFR rs1801133, are associated with pancreatic cancer risk. Among 12 CDKN2A/B SNPs, only the co dominance model of CDKN2A/B rs2518719 could predict the risk of pancreatic cancer, based on Thakkinstian's algorithm (34).

In conclusion, our data showed that CDKN2B was associated with increased risk developing PDAC, indicating the need for further investigations in larger populations and functional studies to confirm the potential value of the emerging marker for PDAC.

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