

Direct Sequencing of Cyclooxygenase-2 (COX-2) Revealed an Intronic Variant rs201231411 in Iranian Patients with Pancreatic Cancer

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ABSTRACT

BACKGROUND

There are hoarding documents for the biological importance of cyclooxygenase-2 (COX-2) in pancreatic carcinogenesis. We aimed to thoroughly investigate the DNA sequence variations of whole COX-2 exons in a large case-control study of pancreatic cancer by direct sequencing.

METHODS

The entire exonic regions of COX-2 including 10 exons were sequenced in the germline DNA of 96 patients with pancreatic cancer. Selected variants within exons six to seven (E6E7) amplicon from the test panel were genotyped in 96 controls.

RESULTS

The COX-2 gene was demonstrated to be genetically conserved. Four missense mutations were found in three cases. However the common variant c.724-10_724-7delATTT (rs201231411) that is located in intron 6, showed significant difference between cases and controls (21 [21.9%] vs 11 [11.5%], $p=0.05$).

CONCLUSION

This study determined that COX-2 has a conservative sequence, which is required for its enzymatic activity and supports the important role of this enzyme's expression in pancreatic cancer rather than any changes in its activity. The effect of intronic variant rs201231411 on COX-2 expression could be analyzed in future studies.

KEYWORDS

Cyclooxygenase-2 (COX-2); Pancreatic cancer; Genome sequencing

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INTRODUCTION

Recent developments in cancer research have discovered the effect of inherited genomic mutations in cancer risk and response to treatment.¹ Pancreatic cancer has been characterized by accumulation of a variety of genomic mutations involve in the progression of normal cells to pre-malignant lesions and then to fully invasive tumor cells.²

Research activities in the field of molecular biology of pancreatic ductal adenocarcinoma (PDAC) have increased in the past decades. The incidence rate of this cancer is rising in Iran and it is usually di-

agnosed in advanced stages with no identified risk factors.³ There are some evidences suggesting that the systemic inflammatory responses associated with chronic pancreatitis play a role in etiology of pancreatic cancer.^{4,5} This suggests that genetic alterations which result in increased inflammatory response and thus chronic inflammation could potentially increase the risk of cancer.^{6,7} One of the genes that code a pro-inflammatory protein is Cyclooxygenase-2 (COX-2), also known as prostaglandin-endoperoxide synthase 2 (PTGS2).⁸ This protein converts arachidonic acid to prostaglandins and other eicosanoids.⁹ There are two cyclooxygenase isoforms, COX-1 and COX-2, whose expressions are regulated by cytokines at both the transcriptional and post-transcriptional levels and are targets of non-steroidal anti-inflammatory drugs (NSAIDs).^{8,10} It is suggested that over-expression of COX-2 results in carcinogenesis through inflammation.¹⁰ In fact, the importance of COX-2 in the etiology of gastrointestinal (GI) cancers is well documented.¹¹ The increased amounts of COX-2 protein are found in more aggressive tumors with poorer prognosis such as pancreatic cancer.¹² Several works have focused on investigating the expression level of COX-2 and risk of PDAC. However, the role of sequence variation and mutations of the COX-2 gene that may contribute to the risk of PDAC are still unknown.¹² The COX-2 gene is 8 kb in length and has 10 protein-coding exons. This gene is located on the human chromosome 1q25.2–q25.3.⁹

In this study, we analyzed the genomic alteration in the entire exons 1 to 10 of COX-2 gene to investigate the relationship between COX-2 mutations and the risk of pancreatic cancer.

MATERIALS AND METHODS

Study Population and Sample Collection

Ninety six patients diagnosed with pancreatic cancer were recruited at the Department of Gastroenterology of Shariati Hospital, Tehran, Iran, during July 2010 and March 2012. The inclusion criteria consisted of confirmed diagnosis of pancreatic cancer confined to the pancreas both histologically and clinically. In order to provide a matched control cohort, 96 healthy controls were included from a ran-

dom sampling from the Health Examination Cohort. All individuals completed a self-administered questionnaire and blood samples were collected by venipuncture into EDTA glass tubes. The participants received genetic counseling for relatives affected at the Digestive Disease Research Institute and all provided written informed consent.

DNA Extraction

Blood samples were centrifuged at 2,000g for 10 minutes and the buffy coats were separated manually. DNA was extracted using the Gentra Puregene kit according to the manufacturer's recommendation (Qiagen, Alameda, CA, USA).

COX-2 Amplification and Sequencing

The COX-2 sequence was obtained from NCBI build 37 reference assembly with NCBI Reference Sequence: NG_028206.1 (PTGS2 prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) [Homo sapiens (human)]). Primers for amplifying all 10 COX-2's exons were designed by Primer 3 software [10]. The primer sequences are shown in table 1. All PCRs were done in total volume of 25 ul comprising 19.8 ul H₂O, 2.5 ul 10x reaction buffer, 0.5 ul dNTP (10 mM), 0.2 ul Taq DNA polymerase (Qiagen Hot Start kit) and 0.5 ul of each forward and reverse primers (20 uM). The PCR started with 10 min at 96 °C and followed by 45 cycles of 30 sec at 96 °C, 30 sec at T_m of 60-63 °C and 30 sec at 72 °C and was ended by 10 min at 72 °C.

The DNA fragments of PCR products were sequenced by the Big Dye Terminator Cycle Sequencing kit on an ABI 3500xl DNA Analyzer (Applied Biosystems Inc.). All sequences were matched to the COX-2 reference sequence using Phred & -Phrap software. In the second step, selected variants which were located within two exons 6 to 7 in the test panel were genotyped in the control group using direct sequencing.

Statistical Analysis

Different characteristics of cases and controls were compared using independent t-test and Chi-square test as appropriated. Logistic regression analysis was used for adjusting the comparison of mutation frequencies of cases and controls for potential confounding

Table 1: The COX-2 (PTGS2) primer sequences by Primer3Plus

Name	Sequence	Amplicon Size (bp)
PTGS2E1F	TGTA AACGACGGCCAGTtaaggggagaggaggaaaa	376
PTGS2E1R	CAGGAAACAGCTATGACCaggaggtcagagcggaaact	
PTGS2E2E3F	TGTA AACGACGGCCAGTaaaaattgtatttccatgactacctat	856
PTGS2E2E3R	CAGGAAACAGCTATGACCtcttgctgatccaaatccaa	
PTGS2E4F	TGTA AACGACGGCCAGTttttggagttacattcaacctcag	312
PTGS2E4R	CAGGAAACAGCTATGACCcaatgcagcccgtcttataag	
PTGS2E5F	TGTA AACGACGGCCAGTcagtcaccatctcctttcttga	304
PTGS2E5R	CAGGAAACAGCTATGACCagcccttgactatgatttgg	
PTGS2E6E7F	TGTA AACGACGGCCAGTaaagaaaaactcaacagcaacaaa	819
PTGS2E6E7R	CAGGAAACAGCTATGACCTTGCACATAATCTTCAATCACAA	
PTGS2E8F	TGTA AACGACGGCCAGTtgtgtaaatgaaactcacaca	886
PTGS2E8R	CAGGAAACAGCTATGACCcccagcggagacaattttta	
PTGS2E9F	TGTA AACGACGGCCAGTattgtctccgctgggagttt	343
PTGS2E9R	CAGGAAACAGCTATGACCggctccatctcgaaaagaaa	
PTGS2E10F	TGTA AACGACGGCCAGTgaatcaggggtcaaatggaa	869
PTGS2E10R	CAGGAAACAGCTATGACCTTCAGCATTTTGCCATCTTG	

covariates. Two-sided *p* values were reported, and *p*<0.05 was considered statistically significant. All analyses were done by STATA software (version 12, StataCorp, College Station, TX, USA A).

RESULTS

Study Population

The overall study population included 96 patients with pancreatic cancer and 96 controls. As shown in table 2, the mean age and sex were different between case and controls; therefore, a logistic regression test was used for adjusting the comparison of variant frequencies between cases and controls for age and sex.

COX-2 Sequencing Analysis

Alteration in sequences of all COX-2 exons and two intronic regions (intron 2 and 6) in the group of cases were analyzed compared with the reference sequence. The list of COX-2 nucleotide changes of germline DNA and their frequencies among patients are shown in table 3.

Sequencing of all exons and the adjacent introns in 96 patients with pancreatic cancer revealed four missense mutations in three patients and a deletion in intron 6 (c.724-10_724-7delATTT) in 19 patients. None of the four missense mutations have been re-

ported previously in public databases. Gln at codon 227 and Arg at codons 226 and 442 are highly conserved in different species and any changing of these amino acids is predicted to be pathogenic by in silico tools like mutation taster.¹³

For further investigating the differences between frequencies of these variants among cases and controls and their possible association with risk of developing pancreatic cancer, the three missense variants at codon 226 and 227 and also the intronic variant were genotyped among 96 healthy controls by direct sequencing of the exons 6 and 7 of COX-2 gene. None of the three missense variants was seen among controls, but the intronic variant (c.724-10_724-7delATTT) was seen in 11 controls indicating an association with pancreatic cancer with a borderline significance (19.7% vs 11.5%, OR=2.16, 95% CI: 1.00-4.78, *p*=0.05). After adjusting the comparison for covariates presented in table 4, the same trend was still observed (OR= 2.15, 95% CI: 0.88-5.27, *p*=0.09).

DISCUSSION

Previous studies offered a major role for enzymes that are involved in the prostaglandin-synthesis pathway (such as COX-2), in the initiation and progression of series of cancers.^{14,15} The activation of COX-2 most often occurs during inflammation which

Table 2: Characteristics of study participants

Variable	Cases, N=96	Controls, N=96	p-value
Age (years)	63.96±9.7	55.34±15.6	<0.001
Gender, M/F	64/32	47/49	0.013
Tobacco	8 (8.3)	16 (17.4)	0.063
Alcohol	7 (7.4)	2 (2.2)	0.097
Opium	20 (20.8)	23 (24.7)	0.523

Table 3: The characteristics and frequencies of COX-2 identified variants in 96 patients in comparison with each allele MAF in ESP6500

Gene	Amplicon	DNA Change	Protein Change	Mutation Type	Frequency (%)	MAF in ESP6500*	rs#
COX2	E9	c.1325G>A	p.Arg442Lys	Missense	1(1.04)	0	NA
COX2	E6E7	c.678A>C	p.Arg226Ser	Missense	1(1.04)	0	NA
COX2	E6E7	c.680A>C	p.Gln227Pro	Missense	1(1.04)	0	NA
COX2	E6E7	c.681G>T	p.Gln227His	Missense	2(2.08)	0	NA
COX2	E6E7	c.724-10_724-7delATTT		Intronic Deletion	19(19.7)	0.05	rs201231411

*The latest frequencies from the NHLBI Exome Sequencing Project

Table 4: Logistic regression analysis for intronic variant (c.724-10_724-7delATTT)

	OR (95% CI)	p-value
Unadjusted		
c.724-10_724-7delATTT	2.16 (1.00-4.78)	0.050
Adjusted*		
c.724-10_724-7delATTT	2.15 (0.88-5.27)	0.092

* Model was adjusted for age, sex, smoking, alcohol consumption and opium use

is a known etiological factor for pancreatic cancer.¹⁵ The importance of COX-2 in etiology of pancreatic cancer has been supported by application of COX-2 inhibitors which attenuated the tumorigenicity of pancreatic cells.^{16,17} Increased level of this enzyme in patients with pancreatic cancer also associated with short survival in several studies.^{2,16} The mechanism by which COX-2 promotes the tumorigenicity is explained by activation of anti-apoptosis pathway and stimulating the expression of multiple positive regulators of cell cycle and cell migration.¹⁴

In this study we present, for the first time, sequencing and analysis of the whole exons in addition to two intronic regions (intron 2 and 6) of COX-2 in human genome of patients with pancreatic cancer. We scanned COX-2 in the germline DNA of 96 cases of pancreatic cancer. As was estimated, we did not see any loss of function mutations, such as a stop codon

or frame-shift insertions or deletions among exonic regions in our cases. Since COX-2 activity should be elevated for increasing cancer risk and this gene could be considered as an oncogene, then the cancer predisposing mutations in coding region of this gene should result in gain of function. It is possible that the three missense mutations at two adjacent codons (226-227) seen in three pancreatic cancer cases result in increased enzymatic activity of the COX2 protein that could be evaluated in future research. Furthermore, an intronic variant rs201231411 with a borderline significance was more frequent in the germline DNA of cases, compared to controls. This intronic variant has been reported in G1000 samples with a MAF (should be spelled out first) of 0.05 which is consistent with the frequency among our 96 controls. The intronic regions do not translate to amino acid sequences, but they could potentially affect the protein product by changing the splicing site in RNA and therefore the final mRNA product. In other words, this part of human genome harbors important regulatory elements that could affect gene expression.¹⁸ The effect of COX2 c.724-10_724-7delATTT variant on the splicing of the COX2 RNA is unknown and there is no information about mRNA isoform with deletion of exons around this mutation. Although the association of nucleotide substitution in the promoter region of COX-2 with the increased

expression level of this enzyme and thus higher risk of AML should be spelled out first as well as colon cancer has been reported previously.^{19,20} There is no data about variant in this region and over expression of COX-2 in pancreatic cancer that might be useful to be assessed in future.

In summary, this study determined that COX-2 has a conservative sequence and germline mutations in COX-2 exons are rare to be associated with pancreatic cancer. However we found a borderline significant difference of intronic deletion c.724-10_724-7 (rs201231411) between patients with pancreatic cancer with the control group. This finding is consistent with the biological function of the introns that control the expression of a particular gene and also support the importance of COX-2 expression and risk of pancreatic cancer that have been investigated previously. Our results also recommend that whole genome sequencing is a valuable tool for understanding variations in the human genome and in common serious diseases in our population.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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