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Novel non-synonymous polymorphisms in the COX-1 gene in Turkish pediatric patients with cardiovascular anomalies

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ABSTRACT. Variation in the gene encoding cyclooxygenase-1 (COX-1) is involved in the process of aspirin resistance. This study investigated the genetic variations in the COX-1 gene. The 4 coding regions of the human COX-1 gene in 90 pediatric patients (median age of 6.5 months, 55% males) with cardiovascular anomalies were screened using DNA sequencing. Twenty coding-region variants causing amino acid substitutions as well as 2 new non-synonymous polymorphisms were identified. All variants were compared with an independent Caucasian population (N = 24 unrelated individuals). Most of the discovered polymorphisms were rare, although some variants resulted in amino acid changes occurring at a frequency >5% (W8R, P17L, Q41Q, Q240Q, D189E, and P188P). In addition, 2 new non-

synonymous polymorphisms (F200L and D189E) were identified. These findings demonstrated novel genetic variants of the human COX-1 gene. Future studies characterizing the functional impact of these variants are warranted.

Key words: COX-1; Gene; Polymorphism

INTRODUCTION

For primary and secondary prophylaxis, pediatric patients with cardiovascular anomalies are administered aspirin to prevent arterial vascular events (Monagle et al., 2008). In children receiving aspirin for diverse indications, incidence of laboratory and clinical aspirin resistance following pediatric cardiac surgery was estimated at 2.3% (Yee et al., 2008) and 10% (Cholette et al., 2010), respectively. Aspirin responsiveness can be influenced by polymorphisms in the gene encoding the cyclooxygenase-1 (COX-1) enzyme, causing amino acid substitutions that could change the specific activity of COX-1 (Halushka et al., 2003).

The COX-1 gene has been localized to chromosome 9q32-q33.3 and has been cloned (Yokoyama and Tanabe, 1989). The gene consists of 11 exons (Funk et al., 1991). Polymorphisms in both coding and non-coding regions of the human COX-1 gene have been identified (Ulrich et al., 2002), and the COX-1 gene has been re-sequenced as part of the Seattle single-nucleotide polymorphism (SNP) Variation Discovery Resource (http://pga.mbt.washington.edu/). In particular, non-synonymous variants encoding amino acid substitutions in the COX-1 protein have been identified (Scott et al., 2002).

We screened the 4 coding regions of the human COX-1 gene in pediatric patients with cardiac anomalies for SNPs, using DNA sequencing. Our primary objective was to identify or confirm the existence of SNPs in the COX-1 gene. Subsequently, we determined the allele frequencies of the observed polymorphisms and discovered 2 new non-synonymous polymorphisms.

MATERIAL AND METHODS

Patient selection

In the presented study, we enrolled 90 pediatric patients (50 males and 40 females), with cardiovascular anomalies, who were candidates for operative surgery. Twenty-four healthy unrelated adults were control subjects. All 114 subjects were Caucasians and from the Aegean region of Turkey.

We sequenced 4 of the 11 exons of the COX-1 gene in 90 patients and identified 2 novel non-synonymous polymorphisms.

DNA extraction and PCR amplification

Genomic DNA was extracted from peripheral blood by using a DNA extraction kit (Invitrogen, Carlsbad, CA, USA) following a standard protocol (Miller et al., 1988). To identify polymorphisms, the coding exons of COX-1 were amplified individually. The primer sequences, $MgCl_2$ concentrations, and annealing temperatures for each amplicon are listed in

Table 1. Each reaction was amplified with a 25-µL mixture containing 2.5 µL 10X PCR buffer with variable $MgCl_2$ concentrations [Applied Biosystems (ABI), Foster City, CA, USA], 2.5 mM dNTPs, 10 pM of each primer, 0.7 U Taq DNA polymerase (Roche, Mannheim, Germany), and 100 ng genomic DNA. Thermal cycling was performed using a thermal cycler (Applied Biosystems 9700) as follows: initial denaturation for 12 min at 95°C; 35 cycles at 94°C for 30 s, 62°C (exons 2 and 3) or 60°C (exons 6 and 7) for 30 s, 72°C for 30 s; and a final extension for 10 min at 72°C.

Table 1. Primers and PCR conditions for COX-1 sequencing.

Exons	Primers	$MgCl_2$ (mM)	Annealing temperature (°C)	Amplicon length (bp)
2	Cox1-2112F: 5'-ATGAGCCGTGAGTGCGACCC-3' Cox1-2462R: 5'-GCTTCAGGGAGCCCCCATC-3'	1.2	66	351
3	Cox1-8816F: 5'-GAGCTGCGACTTAAGTCCAT-3' Cox1-9123R: 5'-GGAAGTTAGGGTCTAGGAGA-3'	1.5	62	308
6 and 7	Cox1-12346F: 5'-GCAGCAAGATCCAGATAGGA-3' Cox1-12872R: 5'-CTCTCAGGACATGACCCAGA-3'	1.0	62	527

DNA sequencing

All the amplified products were sequenced bidirectionally using BigDye Terminator Mix version 3.1 (Applied Biosystems), according to manufacturer instructions, and were analyzed on an ABI-3100 Genetic Analyzer (ABI). Nucleotide sequences were compared with the published COX-1 gene cDNA sequence in the PARSESNP database (Greene E and Taylor N; http://www.proweb.org/parsesnp).

The study was approved by the Ege University Institutional Review Board and conducted according to the ethical principles in the Declaration of Helsinki and Good Clinical Practice guidelines. A written informed consent was obtained from parents and each individual.

Statistical analyses were performed with the SPSS statistical package (version 17.0, SPSS, Chicago, IL, USA). All numerical data are expressed as median (interquartile range) values or proportions. Categorical variables are presented as number of cases (percentage) and are compared using the chi-square test. Two-sided P values of less than 0.05 were regarded as significant.

RESULTS

The median age was 6.5 months, with an interquartile range of 1 day to 16 years. Of these, 8 (8.8%) patients had vascular anomalies such as patent ductus arteriosus (2.2%), aberrant subclavian artery (2.2%), and aortic coarctation (4.4%). Eighty-two (91.2%) patients had diverse cardiac anomalies (Table 2). The median age was 31 years (18-43) in the control group.

Sequencing of exons 2, 3, 6, and 7 of the COX-1 gene from the 90 pediatric patients of known ethnicity identified 20 SNPs. Of these, 7 SNPs were located in exon 2, 8 were located in exon 3, and 5 were located in exons 6 and 7. The most frequent SNP was W8R in both groups (95.6 and 87.5%, respectively; P = 0.14).

In all subjects, the total number of amino acid substitutions was 143 (73%), and the total number of SNPs was 54 (27%); in the study group, the frequencies were 72 and 28%, respectively. The most frequent amino acid substitutions were tryptophan to arginine (W8R) (93.9%) and proline to proline (P188P) (28.1%) (Table 3).

Table 2. Operated patients with congenital cardiovascular anomalies.

Cardiac and vascular anomalies	N = 90 (%)
Single ventricule	14 (15.6)
ASD	13 (14.5)
TGV	11 (12.3)
Tetralogy of Fallot	11 (12.3)
VSD	7 (7.7)
Truncus arteriosus	4 (4.5)
Aortic coarctation	4 (4.5)
Subaortic discrete membrane	3 (3.3)
VSD + ASD	2 (2.2)
Aberrant subclavian artery	2 (2.2)
ASD + PAPVR	2 (2.2)
Pulmonary stenosis	2 (2.2)
A-V canal defect	2 (2.2)
VSD + PDA + PFO	2 (2.2)
PDA	2 (2.2)
TAPVR	2 (2.2)
ASD + VSD + TGV	2 (2.2)
Aortic valve stenosis	1 (1.1)
IHSS + mitral valve insufficiency	1 (1.1)
Hipoplastic left heart	1 (1.1)
VSD + pulmonary atresia	1 (1.1)
Cor triatriatum sinistrum	1 (1.1)

ASD = atrial septal defect; TGV = transposition of the great vessels; VSD = ventricular septal defect; PAPVR = partial anomalous pulmonary venous return; A-V = atrio-ventricular; PFO = patent foramen ovale; PDA = patent ductus arteriosus; TAPVR = total anomalous pulmonary venous return; IHSS = idiopathic hypertrophic subaortic stenosis.

Table 3. Distribution of amino acid substitutions and polymorphisms in all subjects.

Exons	AA substitution	Codon	NC	AA substitution	N = 114	%
2	Tryptophan/arginine	TGG/CGG	T-C	W8R	107	93.9
	Proline/leucine	CCG/CTG	C-T	P17L	6	5.3
	Leucine/proline	CTG/CCG	T-C	L13P	2	1.8
	Leucine/leucine	CTC/CTG	C-G	L23L	1	0.9
	Proline/arginine	CCG/CGG	C-G	P18R	1	0.9
	Leucine/tyrosine	CTG/GTG	C-G	L15Y	2	1.8
	Glutamine/glutamine	CAG/CAD	G-A	Q41Q	7	6.1
3	Proline/proline	CCA/CCC	A-C	P39P	2	1.8
	Arginine/arginine	CGC/CGA	C-A	R53R	1	0.9
	Arginine/serine	CGC/AGC	C-A	G44G	1	0.9
	Cysteine/tryptophan	TGC/TGG	C-G	C58W	2	1.8
	Isoleucine/methionine	ATC/ATG	C-G	I45M	1	0.9
	Arginine/histidine	CGC/CAC	G-A	R53H	1	0.9
	Glycine/glycine	GGC/GGT	C-T	G62G	1	0.9
	Glutamine/glutamine	CAG/CAA	C-A	Q240Q	6	5.3
6 and 7	Arginine/leucine	CGT/CTT	G-T	R239L	2	1.8
	Phenylalanine/leucine	TTT/TTG	T-G	F200L	1	0.9
	Aspartic acid/glutamic acid	GAC/GAG	C-G	D189E	20	17.5
	Lysine/threonine	AAG/ACG	A-C	K185T	1	0.9
	Proline/proline	CCT/CCC	C-C	P188P	32	28.1

AA = amino acids; NC = nucleotide change.

All variants were compared with an independent Caucasian population (N = 24 unrelated individuals) (Table 4). Two new SNPs were detected, F200L and D189E, encoded in exons 6 and 7, respectively. F200L was observed in 1 patient (1.1%) and only in the study group (P = 0.60). The frequency of the D189E SNP was 18.9 and 12.5% in the study and control groups, respectively (P = 0.46).

Table 4. Comparison of polymorphisms.

Exons	Codon		Amino acid substitution	Variant allele frequency		
				Study group [N = 90 (%)]	Controls [N = 24 (%)]	P
2	TGG/CGG	HOM	W8R (Trp8Arg)	86 (95.6)	21 (87.5)	0.14
	CCG/CTG	HET	P17L (Pro17Leu)	6 (6.7)	0	0.19
	CTG/CCG	HET	L13P (Leu13Pro)	2 (2.2)	0	0.46
	CTC/CTG	HET	L23L (Leu23Leu)	1 (1.1)	0	0.60
	CCG/CGG	HET	P18R (Pro18Arg)	1 (1.1)	0	0.60
	CTG/GTG	HET	L15Y (Leu15Tyr)	1 (1.1)	1 (4.2)	0.31
3	CAG/CAA	HET	Q41Q (Gln41Gln)	5 (5.6)	2 (8.4)	0.61
	CCA/CCC	HET	P39P (Pro39Pro)	2 (2.2)	0	0.46
	CGC/CGA	HET	R53R (Arg53Arg)	1 (1.1)	0	0.60
	GGC/GGG	HET	G44G (Gly44Gly)	1 (1.1)	0	0.60
	TGC/TGG	HET	C58W (Cys58Trp)	2 (2.2)	0	0.46
	ATC/ATG	HET	I45M (Ile45Met)	1 (1.1)	0	0.60
	CGC/CAC	HET	R53H (Arg53His)	1 (1.1)	0	0.60
	GGC/GGT	HET	G62G (Gly62Gly)	1 (1.1)	0	0.60
6 and 7	CAG/CAA	HOM	Q240Q (Gln240Gln)	6 (6.7)	0	0.19
	CGT/CTT	HOM	R239L (Arg239Leu)	2 (2.2)	0	0.46
	TTT/TTG	HET	F200L (Phe200Leu)	1 (1.1)	0	0.60
	GAC/GAG	HET	D189E (Asp189Glu)	17 (18.9)	3 (12.5)	0.46
	AAG/ACG	HET	K185T (Lys185Thr)	1 (1.1)	0	0.60
	CCT/CCC	HET	P188P (Pro188Pro)	31 (34.4)	1 (4.2)	0.002

HOM = homozygote; HET = heterozygote.

Most of the discovered polymorphisms were rare, although some variants resulting in amino acid substitutions occurred at a frequency >5%: W8R, P17L, Q41Q, Q240Q, D189E, P188P. The polymorphisms found only in the study group corresponded to P17L, L13P, L23L, P18R, P39P, R53R, G44G, C58W, I45M, R53S, G62G, Q240Q, R239L, F200L, and K185T. In control subjects, W8R, L15Y, Q41Q, D189E, and P188P polymorphisms were observed. Only the P188P amino acid substitution was statistically more frequent in the study group than in the control group (34.4 *vs* 4.2%, respectively, P = 0.002).

DISCUSSION

The present study reports the screening of the coding region of the COX-1 gene, identification of polymorphisms, their allele frequencies in congenital anomalous patients and healthy individuals. We here report on 2 new non-synonymous polymorphisms resulting in amino acid substitutions. To our knowledge, this is the first report of allele frequencies on COX-1 polymorphisms in pediatric patients with cardiovascular anomalies.

COX-1 is the primary target of aspirin. Genetic variants that affect enzyme function, or the protein's interaction with aspirin, could alter an individual's drug response. Polymorphisms may increase the risk of cardiovascular disease or aspirin resistance (Halushka et al., 2003). It is unclear whether common variants affect the structure and function of the mature enzyme. Thus, it is believed that the differences seen in the function of COX-1 most likely result from genetic variation in the regulatory or signal peptide region of the gene or from variation in platelet development and aging. Effects of such variation would hypothetically affect either the amount of COX-1 protein or the location of COX-1 protein in the cell (Halushka et al., 2003; Lee et al., 2007). COX-1 structure-function studies suggest that several amino acid substitutions with modest influence on arachidonic acid binding to the COX-1 active site can alter the catalytic efficiency and metabolite profile of COX-1-mediated arachidonic acid

metabolism (Thuresson et al., 2001). Perhaps these variants could reduce overall metabolic efficiency by altering the interaction between arachidonic acid and the active site. Functional studies aid in the interpretation of genetic epidemiological studies evaluating associations between COX-1 gene polymorphisms and the risk of diseases that involve altered COX-1-derived prostaglandin synthesis, such as cardiovascular and cerebrovascular disease (Lee et al., 2008; Lordkipanidzé et al., 2011).

Few non-synonymous coding polymorphisms in the COX-1 gene were discovered, and most of these were rare (<5%), based on preliminary allele frequency determinations. We were not able to confirm the 2 new non-synonymous polymorphisms reported in the PARSESNP database (F200L and D189E).

In summary, we reported 2 new polymorphisms in the COX-1 gene and showed the allele frequencies of the new and previously described variants for pediatric patients with cardiovascular anomalies. Because the COX-1 enzyme is important in the pharmacology of aspirin, genetic polymorphisms that alter enzyme function are of interest. Further studies of the functional impact of the 2 new polymorphisms on aspirin metabolism are needed.

While the finding of novel polymorphisms in the COX-1 gene is potentially interesting, there are certain limitations. This is a partial screening of the COX-1 gene-coding regions. Although the patient population was given aspirin to prevent arterial vascular events, the functional impact of polymorphisms with respect to the aspirin-resistance in patients with polymorphisms was not investigated.

Conflicts of interest

The authors declare no conflict of interest.

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