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Genetic variability of the distal promoter of the ST2 gene is associated with angiographic severity of coronary artery disease

Andy Tsapaki · Apostolos Zaravinos · Stavros Apostolakis · Konstantinos Voudris · Konstantina Vogiatzi · Georgios E. Kochiadakis · Demetrios A. Spandidos

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Abstract Genetic polymorphy of the distal promoter region of the ST2 gene influences transcriptional activity and susceptibility to atopic dermatitis and asthma. Based on the inflammatory background of atherosclerosis we hypothesized that ST2 distal promoter genetic polymorphy could also affect susceptibility to coronary artery disease (CAD). To test our hypothesis we performed direct sequencing of a 825 bp locus of the distal promoter -with previously reported significant polymorphy in 63 angiographically diagnosed CAD patients and 63 age and sex matched controls with negative coronary angiography. We identified 13 polymorphisms spanning this region two of which (−27307 T/A and −27614 C/A) had allele frequencies grater than 0.05. We further genotyped 111 subjects by applying allele-specific real-time PCR for the −27307 T/A and 27614 C/A polymorphisms, thereby increasing our sample to 129 CAD patients and 108 age- and sex-matched controls. We identified no phenotype–genotype interactions between cases and controls. However, among case subjects the severity of CAD expressed as a mean number of diseased vessels was greater in −27307 A allele carriers and either allele carriers (−27614 A or −27307 A) than in non-carriers (2.56 ± 0.73 vs. 1.83 ± 0.84, adjusted $P = 0.027$; 2.47 ± 0.74 vs. 1.8 ± 0.83, adjusted $P = 0.023$). Additionally, either allele carriers (−27614 A or −27307 A) were significantly more common in the multi-vessel disease group (n = 54) than in the single-vessel disease group (n = 75). In conclusion, we reported two new polymorphisms in the distal promoter region of the ST2 gene that possibly influence susceptibility to severe CAD. The functional impact of these polymorphisms remains to be determined.

Keywords Interleukin 33 · ST2 · Polymorphisms · Coronary artery disease

Introduction

IL-1 cytokines share a common -trefoil structure and form a complex network of several naturally occurring antagonists and decoy receptors regulating their highly pro-inflammatory properties [1]. IL-33 is the most recently discovered member of the IL-1 cytokine family. It has been identified as a specific ligand for the ST2L receptor [2]. The ST2 gene is a member of the IL-1 receptor family, producing a secreted form and a transmembrane form, soluble ST2 (sST2) and ST2L, respectively [93–95]. Activation of ST2L by IL-33 stimulates intracellular signaling pathways, leading to the activation of NF-κB and consequently the induction of a T helper cytokine response. Soluble ST2 directly bound to IL-33 and suppressed activation of NF-κB. It is therefore believed to be a natural inhibitor of IL-33 signaling [3].

Recently, direct sequencing of the entire ST2 locus revealed that genetic polymorphy of the distal promoter region of the ST2 gene influences its transcriptional

Andy Tsapaki and Apostolos Zaravinos contributed equally to this study.

A. Tsapaki · A. Zaravinos · S. Apostolakis · K. Voudris · K. Vogiatzi · D. A. Spandidos (✉)
Laboratory of Clinical Virology, Faculty of Medicine, University of Crete, Heraklion, Crete, Greece
e-mail: spandidos@spandidos.gr

G. E. Kochiadakis
Department of Cardiology, University Hospital, Heraklion, Crete, Greece

activity. A common polymorphism in this region has been associated with susceptibility to atopic dermatitis and asthma [4, 5]. Based on the inflammatory background of atherosclerosis we hypothesized that ST2 distal promoter genetic polymorphy could also affect susceptibility to coronary artery disease (CAD). To test our hypothesis we performed direct sequencing of this locus in angiographically diagnosed CAD patients and age and sex matched controls with negative coronary angiography. We further explored phenotype–genotype interactions of genetic variations with clinical relevance (frequency > 0.05) by applying allele-specific real-time PCR.

Materials and methods

Patients and controls

From a DNA Bank that included 412 CAD patients and 270 angiographically evaluated non-CAD controls, 129 case-samples were selected and 108 age- and sex-matched controls. Samples were collected over a 2 year recruitment period from the Cardiology Department of University Hospital of Crete. Controls were selected to have at least 1 major CAD-predisposing factor (hypertension, diabetes mellitus, smoking, dyslipidemia and family history of CAD). All study participants were Caucasian Greek residents. Demographic and clinical characteristics of cases and controls are presented in Table 1. Informed consent was obtained from all the individuals participating in the study. Study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by a priori approval by the ethics committee of the University Hospital of Crete.

Table 1 Demographic and clinical characteristics of patients and controls

Median age (range):	Cases 64 (45)		Controls 64 (53)	
	n	%	n	%
Sex male	105	81	90	83
Diabetes	43	33	15	14
Hypertension	83	64	47	44
Dyslipidemia	113	88	50	46
Smoking	83	64	57	53
Family history of CAD	28	22	22	20
Multivessel disease	54	42		
Single vessel disease	75	58		
Total	129		108	

Values refer to number of patients (%), or as indicated

Clinical definitions

Clinical definitions of smoking, hypertension, diabetes, dyslipidemia and positive family history have been previously described [6, 7]. CAD was classified as 1, 2 or 3 vessel disease based on the number of major epicardial vessels with $\geq$70% lumen stenosis. Patients were also divided into single vessel disease group and multi-vessel disease group if one or more than one vessels were affected respectively.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from blood samples using the standard phenol/chloroform and ethanol precipitation protocol. Direct sequencing of the distal promoter region of the ST2 gene was successfully performed in 63 CAD patients and 63 age and sex matched controls. Polymerase chain reaction (PCR) was used to amplify the promoter region. A pair of primers framing a 825 bp fragment ranging from −27750 to −26925 in the gene sequence was applied. The primer pair sequences were as follows: Forward: 5′-ATCCTTAGGCCTCTTCTCATCT-3′ and Reverse: 5′-GCT GCATTGCTTTTTAT-3′. PCR reactions were performed in a total volume of 25 μl containing 5 μM of 5× Green GoTaq® Reaction Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTPs), 0.6 μM primers, 0.6 units of GoTaq Flexi DNA polymerase (Promega, Madison, USA) and 100 ng of genomic DNA. The PCR products were resolved on 2% agarose gel, photographed on an ultraviolet light transilluminator, and excised and processed with the Qiaquick PCR purification kit (Qiagen Inc., Crawley, UK). The sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in a 10-μl volume containing purified PCR product and the sequencing primer. The temperature conditions set for the sequencing reactions were 96°C for 2 min followed by 25 cycles at 96°C for 30 s, 54°C for 10 s, and 60°C for 4 min. The reaction products were precipitated with 2-propanol, washed with 75% ethanol, re-suspended in 25 μl water, and loaded onto an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems), as previously described [8, 9].

The sequences were double-checked by the separate application of the forward and reverse primers. Successful, sequencing was performed in 63 patients and 63 controls.

Genotyping

The Plexor™ Systems genotyping method is based on allele-specific PCR (ASPCR). To verify the presence of polymorphisms at the ST2 promoter bp −27307 and −27614, we performed a genotyping method based on allele-specific PCR (ASPCR), using the Plexor qPCR System (Promega, Madison, WI), as previously described (Fig. 1) [8, 9].

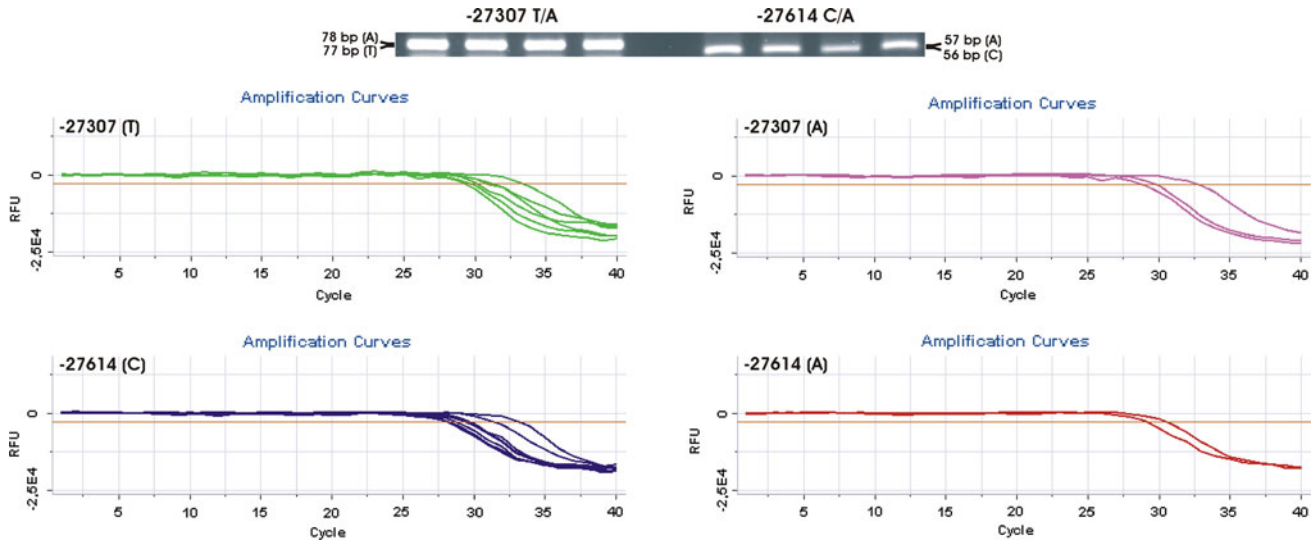

Fig. 1 Amplification curves indicating the polymorphisms detected at the ST2 promoter sites: −27307 T/A and −27614 C/A. The two amplicons had a size of 77–78 and 56–57 bp, respectively

Multiplex real-time qPCR analysis was performed using an Mx3000P Thermal Cycler (Stratagene, La Jolla, CA), and the raw data were further analyzed by the Plexor qPCR software (Promega). Primer sequences that were specific for multiplexing were designed using the Plexor Primer Design Software, and included all the necessary parameters that are required for the Plexor qPCR system. The sequences that we designed for the polymorphism at bp −27614 were: −27614-C: 5′-FAM-iso-dC-AGAGAGTCTCTTCCATGCAT-3′; −27614-A: 5′-HEX-iso-dC-TAGTGCTGCTCTTCCATGAA-3′; and anchor primer: 5′-CAGATTTAACACAGAACTGCCA-3′. As regards the polymorphism at bp −27307, the sequences were: −27307-A: 5′-FAM-iso-dC-ATCGACTAGTACTTACTAAGCCACA-3′; −27307-T: 5′-HEX-iso-dC-GACATCAGTACTTACTAAGCCTCA-3′; and anchor primer: 5′-AGTTATCCTTTCCACAAAACAC-3′. Five microliters of the extracted genomic DNA were used for amplification. The thermal profile was carried out using 2-min incubation at 95°C, and was followed by a second segment comprising an initial denaturation step at 95°C for 5 s and incubation at 50°C for 35 s, and a third segment of denaturation at 95°C for 5 s followed by 40 cycles of 35 s at 60°C. The reaction ended with a Melt-Curve Analysis in which the temperature was increased from 60–95°C at a linear rate of 0.2°C/s. Each sample was analyzed in duplicate.

Statistical analysis

Genotype distributions for each polymorphism were first compared to values predicted by the Hardy–Weinberg equilibrium (HWE) through χ^2 analysis. Haplotypes were calculated and linkage disequilibrium was measured using the classic statistic, disequilibrium coefficient. The correlation of each of the studied polymorphisms to clinical parameters was first evaluated by χ^2 analysis with two degrees of freedom. The extent of the association of each genotype with the disease was initially estimated by Pearson's χ^2 analysis or the Fisher exact test when indicated (expected frequencies ≤ 5). Adjustment for conventional patient risk factors (age, gender, smoking status, diabetes mellitus, hypertension, dyslipidemia and family history) was performed by including these covariates in a logistic regression model. To provide separate odds ratios (ORs) for each genotype, the most common genotype was considered the reference group. Both adjusted and unadjusted ORs are reported in the presence of significant results or borderline significance. With the present sample size, the study had a power of 90% to detect a threefold increase in allele frequencies, assuming a 10% prevalence of the rare allele in the control group and a type I error probability of 0.05.

Numerical values are expressed as the mean ± standard deviation (SD), and differences between the means were compared by the 2-tailed unpaired Student's t test. In all cases, P values < 0.05 were considered statistically significant.

Analyses were performed using SPSS v15 (SPSS Inc., Chicago, IL).

Results

Sequencing analysis

Direct sequencing revealed 13 polymorphisms spanning the 825 bp fragment of the ST2 promoter region (Fig. 2).

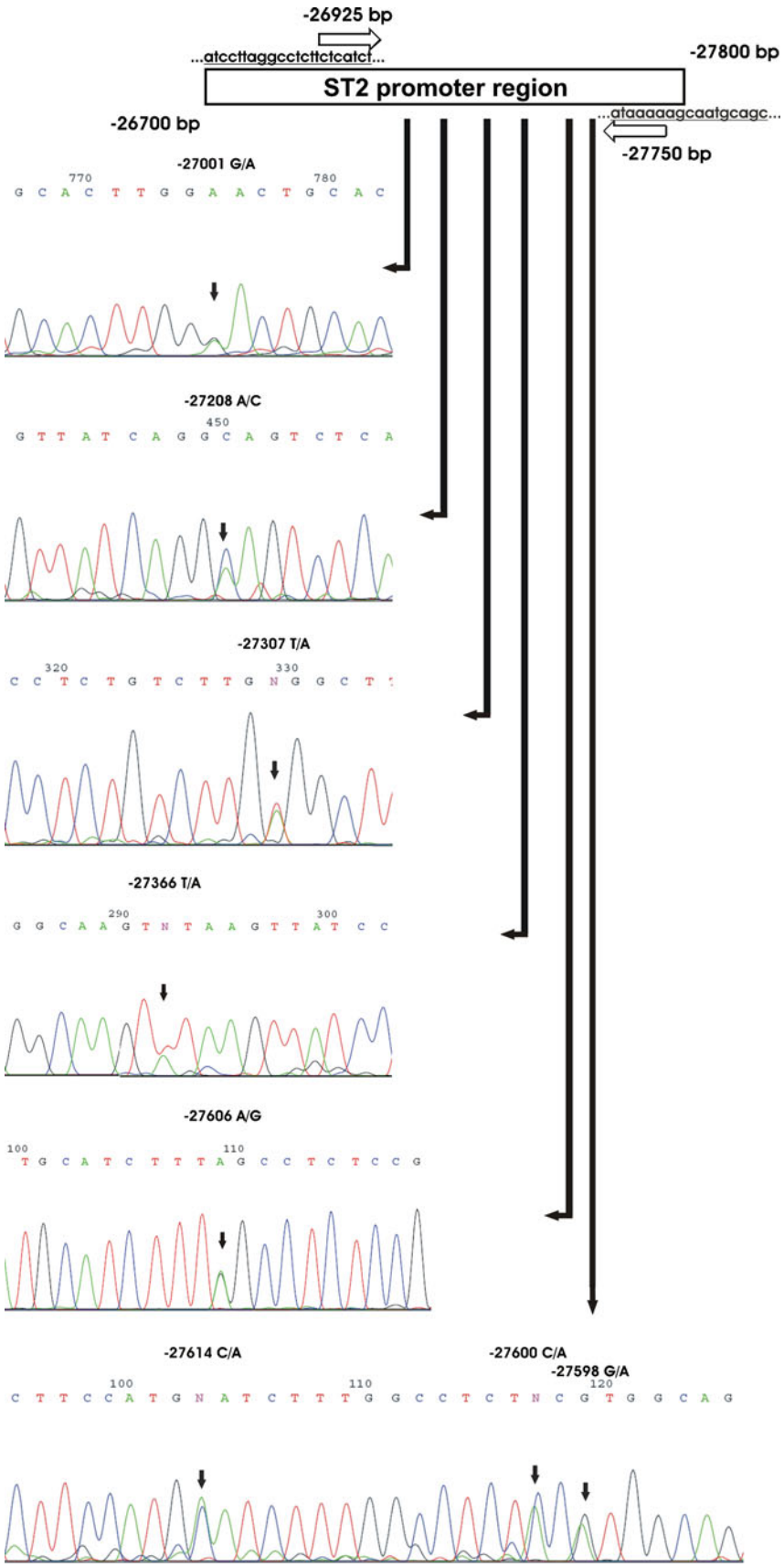

Fig. 2 Representative chromatograms of the most common variations detected in the 825 bp fragment of the ST2 gene distal promoter region, as acquired by DNA sequencing analysis

Table 2 Genotype distribution in cases and controls

Genotype		Cases	Controls
−27307			
TT		120 (93)	98 (92)
TA		9 (7)	10 (8)
AA		–	–
−27614			
CC		116 (90)	96 (89)
CA		13 (10)	9 (11)
AA		–	–
−27307	−27614		
TT	CC	113 (88)	96 (89)
TT	CA	7 (5)	2 (2)
TT	AA	–	–
TA	CC	3 (2)	3 (3)
TA	CA	6 (5)	7 (6)
TA	AA	–	–
AA	CC	–	–
AA	CA	–	–
AA	AA	–	–

Values refer to number of patients (%). No statistical significance in any genotype groups

However, only 2 polymorphisms (−27307 T/A and −27614 C/A) had allele frequencies greater than 0.05 and were considered of potential clinical significance. Due to their extremely low frequency in the studied sample, the 11 remaining polymorphisms were not included in the analysis.

Hardy–Weinberg equilibrium and genetic interaction within the ST2 distal promoter region

Genotype and haplotype frequencies of the −27307 T/A and −27614 C/A SNPs are presented in Tables 2 and 3. Allele frequencies in controls were 0.046 and 0.042, respectively. Genotype frequencies of both polymorphisms were in agreement with those predicted by the Hardy–Weinberg Equilibrium (HWE) in all study groups. Linkage analysis yielded moderate linkage disequilibrium between the two promoter SNPs ($r^2 = 0.4$) while, haplotype analysis revealed 4 potential haplotypes (Table 3). The wild-type allele-containing haplotype (−27307 T/−27614 C or H1) was the most common in both patients and controls accounting of more than 90% of the control gene pool. We did not identify any rare allele homozygotes in our study population.

Genotype–phenotype interactions

Genotype–phenotype interactions were evaluated for each genotype alone, for combined genotypes and also for each of the 4 haplotypes. No statistical significant differences were observed in genotype and haplotype frequencies between cases and controls.

Among case subjects severity of CAD expressed as mean number of diseased vessels was greater in −27307 A allele carriers and either allele carriers (−27614 A or −27307 A) than non-carriers (2.56 ± 0.73 vs. 1.83 ± 0.84, adjusted $P = 0.027$; 2.47 ± 0.74 vs. 1.8 ± 0.83, adjusted $P = 0.023$, respectively), Table 4.

Additionally, either allele (−27614 A or −27307 A) carriers were significantly more common in the multi-vessel disease group (n = 54) than in the single-vessel disease group (n = 75). The latter statistical significance remained unchanged in a multiple logistic regression model where demographic data and conventional CAD risk factors were used as covariates (Table 5).

No associations were established between polymorphisms and either demographic or clinical characteristics of cases and controls.

Discussion

Compelling evidence supports the implication of IL-33/ST2 pathway in various fields of cardiovascular disease [10–14]. It has been proposed that sST2 is a mechanically induced cardiomyocyte protein, and IL-33/ST2 signaling is described as a novel mechanically activated, fibroblast-cardiomyocyte paracrine system, with significant therapeutic potential in the field of myocardial response to overload [10]. Soluble ST2, is thought to participate in the immunologic response related to cardiovascular injury [11, 12] and has been tested as a diagnostic or prognostic marker of patients presenting with acute coronary syndromes, dyspnea or acute heart failure [10]. In the field of

Table 3 Haplotype frequencies in cases and controls

	Haplotype frequencies				LD statistics	
	H1 −27307T/−27614C	H2 −27307T/−27614A	H3 −27307A/−27614C	H4 −27307A/−27614A	D'	r^2
Cases	0.945	0.016	0.016	0.023	0.6	0.4
Controls	0.935	0.01	0.023	0.032	0.75	0.4

Table 4 Correlation of −27307 and −27614 polymorphisms with angiographic severity of coronary artery disease

	Mean number of diseased vessels	Unadjusted 2-tailed significance	Adjusted model	
			t	Significance
−27307				
Carriers	2.56 ± 0.73	0.013	2.24	0.027
Non-carriers	1.83 ± 0.84			
−27614				
Carriers	1.81 ± 0.84	0.04	1.4	0.17
Non-carriers	2.31 ± 0.75			
−27307 or 27614				
Carriers	2.47 ± 0.74	0.004	2.3	0.023
Non-carriers	1.8 ± 0.83			
−27307 and −27614				
Carriers	2.33 ± 0.82	0.181	−0.9	0.3
Non-carriers	1.86 ± 0.85			

Adjusted *P* values have been corrected for age, sex and major predisposing factors of coronary artery disease

Table 5 Correlation of −27307 and −27614 polymorphisms with angiographic severity of coronary artery disease

Rare allele carriers	Single vessel N = 54	Multi vesse N = 75	Unadjusted OR	Adjusted OR	Adjusted *P* value
−27307	1 (1.9)	8 (10.6)	6.5 (0.79–54)	7.4 (0.8–69)	0.08
−27614	2 (3.7)	11 (14.7)	4.6 (1–21)	4.2 (0.8–23)	0.09
−27307 or −27614	3 (5.5)	14 (18.7)	6.2 (1.3–28)	6.6 (1.3–35)	0.026
−27307 and −27614	1 (1.9)	5 (6.7)	3.9 (0.4–34)	3.4 (0.35–33)	0.29

Adjusted *P* values have been corrected for age, sex and major predisposing factors of coronary artery disease

genetic epidemiology there is no previous study assessing the impact of ST2 genetic polymorphy and cardiovascular disease. There are however some interesting indications in different inflammatory-disease settings. Shimizu et al. [5] have assessed the impact of ST2 genetic polymorphy on the ST2/IL33 pathway activity and they established an association between the −26999 A allele in the distal promoter region and atopic dermatitis. Similarly, Ali et al. [4] associated the −26999 A allele with asthma in children.

In the present study we evaluated the effect of ST2 distal promoter polymorphy on susceptibility and angiographic severity of coronary artery disease. We studied a 825 bp region of the distal promoter that based on previous reports included at least 6 polymorphisms two of which were reported functional and common enough to allow us to detect phenotype–genotype interactions at the given sample size. However, in our analysis we identified a completely different polymorphic pattern spanning this particular gene locus including 13 polymorphisms only two of which had allele frequencies exciding 0.05 and none exciding 0.1. These differences could be either attributed to the completely different ethnic and racial groups that where examined in previous studies (Japanese, Australians) or a possible genotyping error [4, 5]. Nonetheless, the derived sequence in our study was identical to the one available in Ensembl (http://www.ensembl.org) and Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) Gene banks. Moreover, we performed direct sequencing in more than 50% of our study participants, a method more reliable than RFLP analysis or allele-specific PCR. Thus, it seems that the ST2 distal promoter region is not only a highly polymorphic site but also a locus with great diversity among different racial and ethnic groups.

In our analysis no functional assays were utilized and we can only speculate a functional consequence of the −27307 T/A and −27614 C/A polymorphisms. Since both variations were associated with more severe CAD in case subjects, these polymorphisms are likely to increase transcriptional activity of the ST2 gene promoting NF-κB activation and subsequently inflammation. However, the lack of functional data is a major study limitation and requires further investigation.

Finally another major study limitation is our sample size. We included a small number of case and control subjects that limited our statistical power and made our study unable to substantiate negative associations. Thus, we can only report positive results and it can still be a great number of phenotype–genotype interactions that remained undetected. Nevertheless, we choose to utilize small but well characterized and strongly matched case and control

samples in order to avoid bias attributed to sample heterogeneity. Moreover, we decided to sequence our site of interest in more than 50% of our sample instead of searching already known polymorphisms with common techniques such as RFLP analysis or allele specific PCR. In fact if we have utilized the latter technique we would have probably found nothing, since none of the previously reported polymorphisms was identified in our sample in clinically relevant frequencies.

In conclusion, we hypothesized that ST2 distal promoter genetic polymorphy affects susceptibility to CAD. To test our hypothesis, we performed direct sequencing/allele-specific real-time PCR of this locus in 129 angiographically diagnosed CAD patients and 108 age- and sex-matched controls with negative coronary angiography. We reported two new polymorphisms in the distal promoter region of the ST2 gene: −27307 T/A and −27614 C/A. Carriers of either polymorphism or the −27307 A allele had angiographically more severe CAD compared to non-carriers. However, the functional impact of these polymorphisms remains to be determined.

Conflicts of interest statement None.

References

1. Apostolakis S, Vogiatzi K, Krambovitis E, Spandidos DA (2008) IL-1 cytokines in cardiovascular disease: diagnostic, prognostic and therapeutic implications. Cardiovasc Hematol Agents Med Chem 6:150–158
2. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA (2005) IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 23:479–490
3. Trajkovic V, Sweet MJ, Xu D (2004) T1/ST2—an IL-1 receptor-like modulator of immune responses. Cytokine Growth Factor Rev 15:87–95
4. Ali M, Zhang G, Thomas WR, McLean CJ, Bizzintino JA, Laing IA, Martin AC, Goldblatt J, Le Souëf PN, Hayden CM (2009) Investigations into the role of ST2 in acute asthma in children. Tissue Antigens 73:206–212
5. Shimizu M, Matsuda A, Yanagisawa K, Hirota T, Akahoshi M, Inomata N, Ebe K, Tanaka K, Sugiura H, Nakashima K, Tamari M, Takahashi N, Obara K, Enomoto T, Okayama Y, Gao PS, Huang SK, Tominaga S, Ikezawa Z, Shirakawa T (2005) Functional SNPs in the distal promoter of the ST2 gene are associated with atopic dermatitis. Hum Mol Genet 14:2919–2927
6. Vogiatzi K, Apostolakis S, Voudris V, Thomopoulou S, Kochiadakis GE, Spandidos DA (2010) Interleukin 8 gene polymorphisms and susceptibility to restenosis after percutaneous coronary intervention. J Thromb Thrombolysis 29:134–140
7. Third Joint Task Force of European Other Societies on Cardiovascular Disease Prevention in Clinical Practice (2003) European guidelines on cardiovascular disease prevention in clinical practice. Eur J Cardiovasc Prev Rehabil 10:1–78
8. Kanellou P, Zaravinos A, Zioga M, Stratigos A, Baritaki S, Soufla G, Zoras O, Spandidos DA (2008) Genomic instability, mutations and expression analysis of the tumour suppressor genes p14(ARF), p15(INK4b), p16(INK4a) and p53 in actinic keratosis. Cancer Lett 264:145–161
9. Zaravinos A, Bizakis J, Soufla G, Sourvinos G, Spandidos DA (2007) Mutations, differential expression of the ras family genes in human nasal polyposis. Int J Oncol 31:1051
10. Sanada S, Hakuno D, Higgins LJ, Schreiter ER, McKenzie AN, Lee RT (2007) IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. J Clin Investig 117:1538–1549
11. Weinberg EO, Shimpo M, De Keulenaer GW et al (2002) Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction. Circulation 106:2961–2966
12. Brown AM, Wu AH, Clopton P, Robey JL, Hollander JE (2007) ST2 in emergency department chest pain patients with potential acute coronary syndromes. Ann Emerg Med 50:153–158
13. Pascual-Figal DA, Ordoñez-Llanos J, Tornel PL, Vázquez R, Puig T, Valdés M, Cinca J, de Luna AB, Bayes-Genis A, MUSIC Investigators (2009) Soluble ST2 for predicting sudden cardiac death in patients with chronic heart failure and left ventricular systolic dysfunction. J Am Coll Cardiol 54:2174–2179
14. Martinez-Rumayor A, Camargo CA, Green SM, Baggish AL, O'Donoghue M, Januzzi JL (2008) Soluble ST2 plasma concentrations predict 1-year mortality in acutely dyspneic emergency department patients with pulmonary disease. Am J Clin Pathol 130:578–584