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differences in the distribution of MIF -173G/C genotypes, alleles or carriage rates between case and control groups in either populations. However, the GG genotype of the MIF SNP rs1007888 was associated with MI in Czech female patients (p=0.027). Conclusions: Taken together with previous reports, our study suggests that particular MIF gene polymorphisms may contribute to MI susceptibility in females.

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☒ **Structured abstract**

☒ **Keywords**

☒ **References are in journal format. References in text and reference list correspond exactly.**

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Short Communication

The Macrophage migration inhibitory factor (*MIF*) gene polymorphism in Czech and Russian patients with myocardial infarction

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Abstract:

Background: Macrophage migration inhibitory factor (MIF) is a cytokine implicated in early and advanced atherosclerosis. The aim of this study was to investigate whether polymorphism of *MIF* gene is associated with myocardial infarction (MI). Methods: Single nucleotide polymorphism (SNP) in *MIF* gene (-173G/C, rs755622) was investigated in Czech (n=219) and Russian (n=240) MI patients and population control from the same geographical areas (Czech, n=137; Russian, n=174). Further, another SNP (rs1007888) located within the 3´ flanking region of the *MIF* gene was investigated in Czech MI patients and control subjects. Results: There were no significant differences in the distribution of MIF -173G/C genotypes, alleles or carriage rates between case and control groups in either populations. However, the GG genotype of the *MIF* SNP rs1007888 was associated with MI in Czech female patients (p=0.027). Conclusions: Taken together with previous reports, our study suggests that particular *MIF* gene polymorphisms may contribute to MI susceptibility in females.

Introduction

Atherosclerotic inflammation of the coronary arteries is a critical process in the pathogenesis of myocardial infarction (MI), an acute manifestation of coronary heart disease (CHD). The initial phase of atherosclerosis is characterized by migration of monocytes and T-cells into the vessel wall, mediated by cytokines and chemokines [1,2] with subsequent release of mediators aggravating progression of atherosclerotic lesions [3]. One of the mediators involved in these processes is the macrophage migration inhibitory factor (MIF), which has already been implicated in the pathogenesis of inflammation [4]. In the context of CHD, there is increasing evidence that MIF participates in atheroma formation and in the progression of vascular disease [5-8]. MIF has been implicated in early plaque development and in advanced complicated atherosclerotic lesions in humans [5]. In line with this observation, MIF plasma levels increase during MI and this may not only reflect the severity of MI, but also its potential importance in the initial MI stage [9]. Data from animal models also support a role for MIF in vascular disease [6]. Recently, an association of the *MIF* gene rs755622 and rs2070766 single nucleotide polymorphisms and corresponding haplotype with coronary heart disease (CHD) in females was reported in the MONICA/KORA Augsburg case-cohort study [10].

Given the biological plausibility of MIF relevance for coronary atherosclerosis, this study investigated whether functional SNP variants located in *MIF* gene are associated with susceptibility to MI and its manifestation. In agreement with current guidelines for genetic association studies [11], this was a two population case control study comprising healthy subjects and patients from two different Caucasian populations: Czechs and Russians.

Materials and Methods

Study population: A total of 770 unrelated individuals were enrolled into the study, in which 219 Czech (Olomouc, Czech Republic; West-Slavonic)[mean age 53.8±8.1 years; 186

males/33 females] and 240 Russian (Novosibirsk, Russia; East-Slavonic)[55.3±9.3; 163/73] patients with MI were compared with 137 Czech [31.3±9.8; 70/67] and 174 Russian [43.5±7.4; 76/98] healthy individuals serving as control populations. MI diagnostic criteria were compatible with those recommended by an international consensus [12]. Informed consent was obtained from all patients and controls and the respective local ethical committees approved the study. The Russian control population was from the MONICA project register (Russia).

Genetic analysis: Two *MIF* gene SNPs [refSNP ID: rs755622 (syn. *MIF* -173 G/C, within promoter region) and rs1007888 A/G (located within the 3´ flanking region)] were genotyped by polymerase chain reaction with sequence-specific primers (PCR-SSP). The primer sequences were: 1) rs755622: allele G, forward 5'CGC CAA GTG GAG AAC AGG, allele C, forward 5'CGC CAA GTG GAG AAC AGC, constant reverse 5'GCA GAG GCA CAG ACG CA and 2) rs1007888: allele A, reverse 5´GAG AAG TAT CGT CCC CAC T, allele G, reverse 5´GAG AAG TAT CGT CCC CAC C, constant forward 5´AGC CAA GGA GAA TGG GAG AT. The amplification internal control and PCR compounds were described elsewhere [13]. The genotyping was checked in 10% of randomly selected samples of all possible genotypes for rs1007888 A/G SNP by TaqMan SNP genotyping assay (Applied Biosystems, Assay ID C_2448251_1) according to the manufacturer's instructions. Complete concordance for rs1007888 genotype calling was observed between the PCR-SSP and TaqMan assays.

Statistical Analysis: The differences in frequencies of *MIF* genotypes/alleles and carriage rates were analysed using the χ^2-test (SPSS Inc, Chicago). The statistical power of the present study was determined as described elsewhere [14]. Maximum-likelihood haplotype frequency for MIF -173 (rs755622) and rs1007888 loci was determined using an Expectation-Maximisation (EM) algorithm for multi-locus data; overall linkage disequilibrium (LD) was

tested by likelihood ratio test (software Arlequin, version 3.0, University of Berne, Switzerland, http://cmpg.unibe.ch/software/arlequin3)[15].

Results and Discussion

1) *MIF* -173 G/C (rs755622) SNP and susceptibility to myocardial infarction in Czech and Russian population

Both Czech and Russian patient and control populations were in Hardy-Weinberg equilibrium with regard to *MIF* -173 G/C genotypes' distribution (p>0.05). Statistical power of the present study to detect the differences in the carriage of the *MIF* -173*C allele corresponding to the odds ratio 2 between the MI cases and healthy controls reached 90.5% for Czech population and 95.7% for Russian population. In both investigated populations, no association was found between the *MIF* -173 G/C genotypes and alleles; minor (C) allele carriage rates also did not differ between cases and controls (Table1).

To explore whether *MIF* -173 G/C SNP may be a possible risk factor for manifestation of MI, patients were subdivided according their gender and the age of the first MI episode (early MI: <50 years). There were no significant differences between patients and healthy controls of either gender in genotype or allele frequencies (p>0.05) in either population. There was no relationship between genotypes/minor C allele and early MI in Czech population. Although a non-significant trend indicating a higher risk for early MI for Russian patients with the *MIF* –173 CC genotype (CC vs. GC/GG<50 yr, p= 0.057) was observed, the frequency of the C allele and its carriage rates did not differ significantly between the groups of patients and controls (<50 yr). Concordantly with these findings, multivariate logistic regression analysis adjusting for age and gender revealed no significant evidence of the association between the *MIF* -173 SNP and the risk of MI in two investigated populations (*MIF* -173 CC vs. GC/GG: p= 0.84 for Czech population and p= 0.52 for Russian population).

Our data obtained from two Caucasian populations of well-defined patients does not confirm reports on German and Asian (Chinese) populations, where the minor *MIF* -173*C allele was implicated as a risk factor for CHD [10,16]. Since the design, genotyping methodology and enrollment criteria in our study complied with current protocols and the data on distribution of *MIF* -173 G/C variants in population control were similar to those from other European populations [e.g. 17], technical problems in our analysis and/or data generation are unlikely. Nevertheless, our study could not be regarded as a direct replication of the above mentioned reports [10,16] due to the difference in the definition of phenotype. While we limited ourselves to the patients with myocardial infarction (MI), the German and Chinese studies utilized subjects diagnosed with CHD as a whole. Phenotype differences may be, therefore, considered as one of the possible explanations for the discordance between the present and previous studies.

Concerning a capacity of our study to replicate previous findings [10,16] it should acknowledged that its statistical power was higher for both Czech and Russian populations than that of a Chinese study [16]. It is noteworthy to add, that even if ethnic differences were the cause of a discrepant role of the investigated SNP in MI in Slavonic Caucasians, the frequency of *MIF* -173*C allele found in Chinese patients was only 8%. Hence the real significance of this genetic variation expressed as Population Attributable Risk is limited. On the other hand, due to the lower absolute numbers of females among our MI patients, our study may not be powered sufficiently to replicate gender - specific association of minor *MIF* -173*C allele with CHD observed in females within MONICA/KORA Augsburg study [10].

2) *MIF* rs1007888 A/G SNP and susceptibility to myocardial infarction in Czech population

During the preparation of this manuscript, Herder et al. reported that allele G of another SNP located within the 3´ flanking region of the *MIF* gene (rs1007888) correlated with the circulating MIF levels and that GG genotype of this SNP was associated with the susceptibility to the disease tightly linked to CHD, namely type 2 diabetes (T2D), in the MONICA/KORA Augsburg study [18]. Although the samples from Russian population were not available for further analysis we extended our investigations to MIF rs1007888 SNP at least in the Czech patients with MI and control population. Importantly, we found that the genotype *MIF* rs1007888 GG was overrepresented in Czech MI patients by comparison with ethnically matched healthy control subjects (p=0.018, Table 1, Figure 1). Furthermore, because rs1007888 GG genotype was previously associated with T2D only in females [18] we performed further subanalysis of our MI patients according to the gender. Interestingly, an association of rs1007888 GG genotype with MI was limited to females (p=0.027, OR=2.99, 95% CI: 1.11-8.05) and was not apparent in males (p=0.16, Figure 1). This association appeared also as the difference in the frequency of *MIF* rs1007888*G allele between female MI patients and control subjects (p=0.02, Table 1) and was independent of the T2D status of MI patients (data not shown).

Although we could provide the evidence for the association of the *MIF* rs1007888 SNP with MI susceptibility in females only in Czech population, the plausibility of this finding may be supported by particular observations from the German MONICA/KORA Augsburg study: 1) The same genotype (*MIF* rs1007888 GG) containing two alleles associated with MIF levels appeared as susceptibility factor for T2D [18] and 2) Association of *MIF* gene variants with CHD was limited to females in German MONICA/KORA Augsburg study [10] concordantly with our present findings. To our knowledge there are, however, no data available that could mechanistically explain the relationship between *MIF* rs1007888 SNP and the levels of circulating MIF. Despite this polymorphism is located

relatively far from the 3´ end of the *MIF* gene translated region, it may interfere with the gene expression or with the stabilisation of transcripts mediated by oestrogen as hypothesised by others [18]. Finally, a possibility that *MIF* rs1007888 SNP is only a marker for the causative functional variant conferring changes in MIF levels could not be excluded.

Because the genotypes on two *MIF* SNPs were available for the Czech population, we were interested if this Slavonic population is similar to other Caucasians with regard to the distribution of *MIF* haplotypes. Importantly, estimated frequencies of MIF -173 (rs755622) G/C and rs1007888 A/G haplotypes in Czech control population (rs755622/rs1007888 haplotype frequencies; GA: 0.47, GG: 0.40, CA: 0.09, CG: 0.05) was quite similar to those observed in the German MONICA/KORA Augsburg study [18]. In our Czech MI patients the frequency of haplotypes possessing G at position rs1007888 was moderately higher (haplotype frequencies; GG: 0.45, CG: 0.07) which is in line with the overrepresentation of *MIF* rs1007888 GG genotype among Czech MI patients.

Study limitations:

Because the data on several parameters known as risk factors for coronary heart disease (smoking, lipid levels, hypertension) were not available for the substantial part of the study population, this retrospective study could not control for them in the multivariate analysis. Because of the difference between the median age of the Czech MI patients and healthy control subjects, confounding by population stratification could not be excluded. Nevertheless, haplotype analysis revealed almost the same distribution of *MIF* haplotypes in our Czech healthy population as observed in another geographically close Caucasian population [18].

In conclusion, our study did not reveal an association of the myocardial infarction or its manifestation with the macrophage migration inhibitory factor promoter *MIF* -173 G/C SNP in Caucasians, at least of those of Slavonic origin. Nevertheless, our data from the Czech

population on another *MIF* rs1007888 SNP may further support a concept of gender – specific implication of *MIF* gene variants in MI susceptibility.

Acknowledgements:

Sources of Funding: The study was supported by the Czech government funding (ME-856 and MSM6198959205). I.T. is the recipient of Visegrad fund fellowship.

List of abbreviations:

CHD - coronary heart disease

MI - myocardial infarction

MIF – macrophage Migration Inhibitory Factor

PCR – polymerase chain reaction

SNP - single nucleotide polymorphism

T2D – type 2 diabetes

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Table 1: Distribution of genotypes, alleles and phenotypes (less common allele carriage) of single nucleotide polymorphisms *MIF* -173 (rs755622, Czech and Russian population) and *MIF* rs1007888 (Czech population including both genders separately) in patients with MI and healthy control subjects (Control).

			Genotype 1	Genotype 2	Genotype 3	Allele 1	Allele 2	Carriage
***MIF* rs755622**		N	GG	GC	CC	G	C	C
Czech population	MI	219	163(74.4)	47(21.5)	9(4.1)	373(85.2)	65(14.8)	56(25.6)
	Control	137	103(75.2)	31(22.6)	3(2.2)	237(86.5)	37(13.5)	34(24.8)
	p values				0.329†		0.620‡	0.874#
Russian population	MI	240	164(68.3)	73(30.4)	3(1.3)	401(83.5)	79(16.5)	76(31.7)
	Control	174	126(72.4)	42(24.1)	6(3.4)	294(84.5)	54(15.5)	48(27.6)
	p values				0.130†		0.716‡	0.371#
***MIF* rs1007888**		N*	AA	AG	GG	A	G	G
Czech population	MI	212	52 (24.5)	103 (48.6)	57 (26.9)	207 (48.8)	217 (51.2)	160 (75.5)
	Control	137	38 (27.7)	77 (56.2)	22 (16.1)	153 (55.8)	121 (44.2)	99 (72.3)
	p values				0.018†		0.070‡	0.503#
	MI males	180	49 (27.2)	85 (47.2)	46 (25.6)	183 (50.8)	177 (49.2)	131 (72.8)
	Control males	70	21 (30.0)	37 (52.9)	12 (17.1)	79 (56.4)	61 (43.6)	49 (70.0)
	p values				0.157†		0.261‡	0.662#
	MI females	32	3 (9.4)	18 (56.3)	11 (34.4)	24 (37.5)	40 (62.5)	29 (90.6)
	Control females	67	17 (25.4)	40 (59.7)	10 (14.9)	74 (55.2)	60 (44.8)	50 (74.6)
	p values				0.027†		0.020‡	0.064#

The data are presented as absolute numbers with percentages in parentheses.

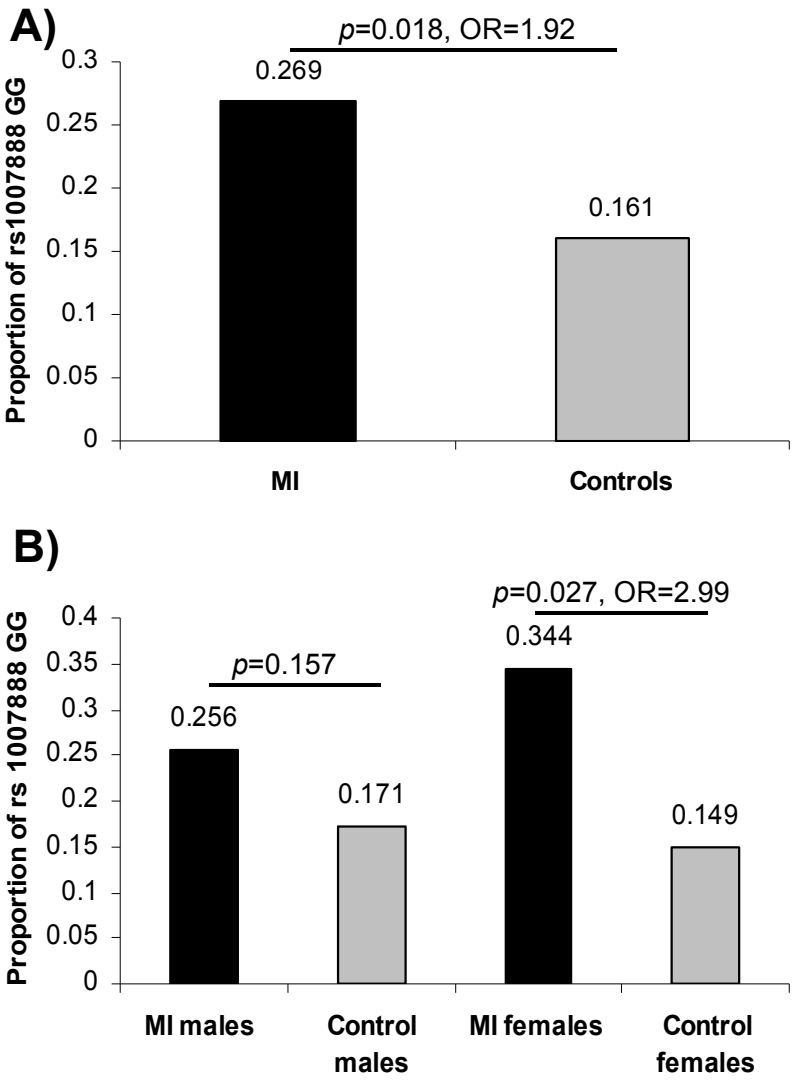
* DNA samples from 7 Czech MI patients were not available for the MIF rs1007888 genotyping.

† *p* values for comparison of Genotype 3 proportion (versus other genotypes) between MI patients and control subjects

‡ *p* values for comparison of Allele 2 proportion (allelic frequency) between MI patients and control subjects

p values for comparison of Allele 2 carriage (phenotype frequency) between MI patients and control subjects

Figure 1:


A)
p=0.018, OR=1.92
0.269
0.161
Proportion of rs1007888 GG
0.3
0.25
0.2
0.15
0.1
0.05
0
MI
Controls
B)
p=0.027, OR=2.99
0.344
p=0.157
0.256
0.171
0.149
Proportion of rs 1007888 GG
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
MI males
Control males
MI females
Control females

Legend to the Figure 1:

Proportion of GG homozygotes of the *MIF* rs1007888 SNP in Czech patients with MI (MI) by comparison with ethnically matched healthy control subjects (Controls) in the whole study population (A) and compared for both genders separately (B).

A) MI *versus* Controls (proportion of GG/other genotypes): p=0.018, OR = 1.92, 95% CI: 1.11-3.32.

B) MI female patients *versus* female control subjects (proportion of GG/other genotypes): p=0.027, OR = 2.99, 95% CI: 1.11-8.05.

The Macrophage migration inhibitory factor (*MIF*) gene polymorphism in Czech and Russian patients with myocardial infarction

Ms. Ref. No. CCA-D-08-00477 – revision 2

Point by point response to the reviewer's comments

Reviewer #1: Much improved manuscript with some new novel positive data linking the gene to the MI phenotype in this revised version. The manuscript could still benefit from a few additions in my opinion given the new data but I am satisfied with the responses to my original comments.

Major:

1.) Please add a few sentences describing the linkage arrangement or haplotypes created by this 2nd SNP in the MIF gene. You can use haploview for this (free to download). Are the reported Caucasian haplotypes seen in your Slavonic population at a similar frequency?

Response:
According to this comment we performed haplotype and linkage disequilibrium analysis of investigated *MIF* SNPs in the Czech population using the Arlequin software (ref. No 15 in the revised manuscript). The procedure is briefly described in the Methods sections (Statistical analysis: page 4, lines 23-25 and page 5, lines 1-2) and the results are provided in the section Results and Discussion (page 8, lines 5-13).
The estimated frequency of *MIF* haplotypes was similar to those reported in another Caucasian (German) population (ref. No 18 in the revised manuscript).

2.) If known, please add a few sentences describing the function of the second SNP. You call it functional but locate it in the 3'UTR. Does it effect mRNA stability?

Response:
We would like to thank the reviewer for this comment. We are aware that an attribute "functional" was not used quite adequately for the *MIF* rs1007888 SNP in the original version of this manuscript, because no direct (mechanistic) evidence of its "functionality" is available at present. We, therefore, omitted a word "functional" in the revised version and described this SNP as "associated with MIF levels" (e.g. page 7, lines 20-21). Further, possible mechanisms responsible for observed relationship of this SNP with MIF levels are suggested (page 7, lines 23-25 and page 8, lines 1-4).

3.) Please add the genotypes and numbers for the 2nd SNP to your Table 1 and give breakdown of n's for male / female.

Response:
The Table 1 has been enlarged with the data for the second SNP from the Czech population including separate numbers (and proportions) for males and females.

4.) Make a second panel broken out by gender for Figure 1. Show OR and P above the bars.

Response:
The Figure 1 was corrected accordingly.

5.) May want to add a limitations section in the Discussion mentioning that you could not control for other risk factors such as smoking and high cholesterol, that the study was retrospective, that the SNP could be in linkage with another relevant SNP, that you could be confounded by population stratification, etc.

Response:
The new paragraph called Study limitations containing issues mentioned by the reviewer was added to the Results and Discussion section (page 8, lines 14-22). A possibility of linkage disequilibrium of rs1007888 SNP with another causative variant is mentioned elsewhere (page 8, lines 3-4).

Minor:
5.) pg 5, change "populations, where minor" to "populations, where the minor"
6.) pg 6, change "enrolment" to "enrollment"
7.) pg 6, change "type 2 diabetes (T2D), in" to "type 2 diabetes (T2D), in the"

Response:
The text of the revised manuscript was corrected according to these minor comments.