Macrophage migration inhibitory factor -173 polymorphism and risk of coronary alterations in children with Kawasaki disease

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Abstract

Objective
To investigate the possible relationship between MIF –173 polymorphism and susceptibility to, and severity of, Kawasaki disease (KD) in a cohort of Italian patients.

Methods
Sixty-nine patients (43 F, 26 M, median age 29 months, range 3-135 months) with KD and 60 sex-matched healthy caucasian children were genotyped for MIF-173. Typing of the MIF gene -173 G/C was performed by PCR and restriction fragment length polymorphism.

Results
Eight out of 69 (12%) KD children were non-responders: 7 required an additional IVIG infusion, while 1 received 2 IVIG infusions and then steroid administration. 9/69 (13%) KD children developed coronary artery abnormalities (CAA) during the second to fourth week of disease, and 4 of them required an additional IVIG infusion. MIF genotyping did not show significant differences between patients and controls. KD patients carrying a MIF -173*C allele developed CAA more frequently than those without MIF- (7/9 77.8% vs. 16/60 26.7%, OR 9.6, 95% CI 1.80–21.2, $p<0.005$).
Non-responders to a single IVIG infusion carried the MIF -173*C allele more frequently than responders (6/8 = 75% vs. 17/61 = 28%, OR 5.1, 95% CI 1.42–22.31 $p<0.014$). In multiple regression analysis, KD patients carrying a MIF -173*C allele were found to have an increased risk of coronary involvement (OR 7.7, 95% CI 1.36–16.1, $p=0.021$).

Conclusions
We showed that children carrying the MIF polymorphism -173*C had a higher percentage of CAA. A potential relationship between a MIF polymorphism and risk of CAA in KD might be considered.

Key words
Kawasaki disease, macrophage migration inhibitory factor, coronary artery abnormalities.

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Introduction

Kawasaki disease (KD), an acute febrile self-limited systemic vasculitis of childhood, represents the most frequent cause of paediatric acquired heart disease in developed countries, leading to coronary complications in 15%–25 % of untreated patients (1). The activation of the immune system seems to be a regulatory player into the immunopathogenesis of KD, as also demonstrated by high serum concentrations of proinflammatory cytokines and chemokines, including tumour necrosis factor-α (TNF-α), IL-1, IL-6, IL-8 (2) and by the reciprocal interactions between monocytes and endothelial cells.

MIF (macrophage migration inhibitory factor) is a protein with hormonal, enzymatic, and proinflammatory functions both in innate and in adaptive immunity (3). Serum levels of MIF have been found to be elevated in patients affected by several inflammatory diseases such as sepsis, acute respiratory distress syndrome, atherosclerosis, juvenile idiopathic arthritis, antineutrophilic cytoplasmic antibody-associated vasculitides, asthma, type 2 diabetes, atopic dermatitis, psoriasis and glomerulonephritis (4). Recently, Lee *et al*. have demonstrated a significant increase of MIF serum levels in the acute phase of KD compared with patients in the subacute stage and with normal and febrile controls (5).

Two MIF polymorphisms have been identified localised on its promoter region: CATT (5-8) at -794 position and MIF -173*C, and several functional observations show that the MIF -173*C allele is associated with higher production of MIF *in vitro* as well as *in vivo* both in healthy subjects and in auto-immune inflammatory diseases, such as rheumatoid arthritis and juvenile idiopathic arthritis (6-11).

Particularly, it has been shown that MIF polymorphism of single nucleotide G to C at position -173 is associated with an increased susceptibility to all JIA forms, with the severity and the response to steroids in systemic JIA and with a shorter response to intra-articular triamcinolone hexacetonide (7-11). Taken together, these data strongly support the potential functional relevance of the MIF -173* polymorphism on inflammatory and immune responses in chronic inflammatory and autoimmune diseases: carrying the C allele seems to predict a poor functional outcome of disease.

We performed a study in order to investigate the possible relationship between MIF -173 polymorphism and susceptibility to, and severity of, KD in a cohort of Italian patients.

Patients

Our study population included 69 patients (43 females and 26 males, median age 29 months, range 3–135 months) discharged from January 1999 to December 2007 from the "A. Meyer" Children's Hospital, Florence, Italy. All fulfilled the diagnosis of KD according to the current criteria (1). All children received the current recommended therapy with intravenous immunoglobulin (IVIG) infusion (2 g/kg) within the first 10 days from the onset of fever and acetylsalicilic acid (50-80 mg/kg during the acute phase of the disease, and 3-5 mg/kg/day thereafter). Children needing more than one IVIG infusion and/or steroid therapy for remission of active disease were considered non-responders .

Sixty sex-matched healthy caucasian children who attended our unit for musculoskeletal symptoms acted as controls, after the exclusion of rheumatic, endocrine, or metabolic diseases.

Cardiac evaluation

Patients with KD underwent electrocardiogram and 2-D echo Doppler on suspicion of KD, before hospital discharge, and after 2, 4 and 8 weeks. According to the degree of coronary involvement, children with coronary artery abnormalities (CAA) were closely followed until normalization of their condition. CAA were defined as the internal lumen diameter greater then 2 standard deviation above the expected mean calculated for body surface area on the basis of the study by De Zorzi and colleagues (12).

Genotyping for MIF-173

Whole blood samples for genotyping were drawn during routine venipunc-

ture. Genomic DNA was extracted from EDTA blood samples using QIAmp DNA Blood kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Typing of the MIF gene -173 G/C was performed by PCR and restriction fragment length polymorphism (RFLP). The presence of C alleles at -173 create an *Alu I* restriction enzyme site. The PCR primer pair comprised forward primer 5' ACT-AAG-AAA-GAC-GAG-AGG-C 3' and reverse primer 5' GGG-GCA-CGT-TGG-TGT-TTA-C 3'.

PCR was performed according to Berdeli *et al*. (13) with few minor modifications. Briefly, PCR was performed in 25 μl vol containing 1 μl of genomic DNA solution, 0,25 mM of each dNTPs, and 10 pM of each specific primers in the presence of gene Amp gold Buffer, and 1 U AmpliTaq Gold Polymerase (Perkin-Elmer, Nonvalk, CT).

PCR was initiated by a 10-min denaturation step at 95°C and completed by a 7-min extension step at 72°C. The temperature cycled for 45 s at 95"C, 45 s at 60°C, and 45 s at 72°C, for *40* cycles. The *G* and C alleles were detected by *AluI* (Fermentas Inc, Hanover, MD) restriction enzyme digestions of the 366-bp product amplified by the PCR primers , followed by a 3% agarose gel electrophoresis. *AluI* cleaves the C allele into two fragments and leaves the *G* allele undigested.

Statistical analysis
An *a priori* power analysis was completed using G Power program (14). Two-tailed *p*-values were employed. Considered previously reported frequency of MIF genotyping in literature (9, 11), a large expected difference was estimated for the sample: the effect size f=0.40, as per Cohen (15). In addition, power was set at 0.95, meaning there would be a 95% probability of reaching statistical significance if the obtained differences were truly present in the population. Results from the power analysis showed that 124 participants in all groups combined would be required. Since the number of subjects carrying a CC genotype was very low, the analysis was performed also comparing frequency data in two groups:

those carrying at least one MIF -173*C allele and those carrying the GG genotype. Frequency data were compared by chi-square test and Fisher exact test with Monte Carlo simulations, when appropriate. The Spearman rank correlation test was used to determine correlation coefficients for different variables (gender, age at diagnosis, fever duration and clinical KD criteria, ESR, CRP, hemoglobin, and platelet count values, timing and number of IVIG infusion, CAA and MIF genotyping). Multiple stepwise regression was performed to determine variables that may correlate independently. The predictors used in the final model, where the presence of CAA was set as dependent variable, were the parameters showing a significant correlation with coronary involvement in the univariate analysis. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated.

Non-parametric tests were used, where necessary, due to the small size of our groups and to the skewness of our data. Levels of *p*<0.05 were considered statistically significant. All analyses were performed on SPSS package for Windows, version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results
All 69 patients received conventional therapy with a single IVIG infusion, but eight of them (12%) were non-responders: seven required an additional IVIG infusion, while one received 2 IVIG infusions and then steroid administration (methyl-prednisolone 30 mg/kg, i.v) due to the persistent activity of the disease with CAA.

Nine children developed CAA (13%) during the second to fourth week of disease, and 4 of them required an additional IVIG infusion. In three patients cardiac abnormalities resolved within the first month of disease; four had normal echocardiograms after 4 months and did not present alterations subsequently, while 2 with persistent dilatations, albeit with reduced diameter, are still being followed.

No evidence of departure from Hardy-Weinberg equilibrium in controls was seen (*p*=0.65).

Statistical analysis for MIF genotyping did not show significant differences

Table I. A. Comparison of MIF -173* G/C allele frequencies (%) in Kawasaki disease (KD) with coronary artery abnormalities (CAA) and without CAA versus controls. Values are the number of subjects tested.

	Kawasaki disease		
Allele	KD with CAA (n=9)	KD without CAA (n=60)	Controls (n=60)
MIF -173* G	2 (22.2%)	44 (73.3%)	46 (76.7%)
MIF -173* C	7 (77.8%)*	16 (26.7%)	14 (23.3%)

There were no significant differences between KD children and controls.
Among KD patients, MIF -173*C allele resulted more frequent in KD with CAA than those without (*p<0.005)

Table I B. Comparison of MIF -173* genotype frequencies (%) in Kawasaki disease (KD) with coronary artery abnormalities (CAA) and without CAA versus controls. Values are the number (%), n=number of subjects examined.

	Kawasaki disease		
Genotype	KD with CAA (n=9)	KD without CAA (n=60)	Controls (n=60)
MIF -173* GG	2 (22.2%)	44 (73.3%)	46 (76.7%)
MIF -173* GC	5 (55.6%)	14 (23.3%)	12 (20.0%)
MIF -173* CC	2 (22.2%)*	12 (3.3%)	2 (3.3%)

There were no significant differences between KD children and controls.
Among KD patients, MIF -173 *CC homozygosis resulted more frequent in KD with CAA than those without (*p<0.004).

between patients and controls, both for allelic and genotypic frequencies. G allele was present in 46/69 (66.7%) KD children compared to 46/60 (76.7%) controls, and C allele in 23/69 (33.3%) KD patients compared to 14/60 (23.3%) controls. The polymorphism distribution resulted as follows: 46/69 GG (66.7%), 19/69 CG (27.5%) 4/69 CC (5.6%) for KD and 46/60 GG (76.7%), 12/60 CG (20%), 2/60 CC (3.3%) for controls.

However, as can be seen in the Table I, KD patients carrying a MIF -173*C allele developed CAA more frequently than those without these alleles (7/9 77.8% *vs*. 16/60 26.7%, OR 9.6, 95% CI 1.80-21.2, *p*<0.005), and the MIF -173 *CC homozygosis resulted more frequent in children with CAA than those without (2/9 CC 22.2% *vs*. 2/60 CC: 3.3%, *p*<0.004). Moreover, nonresponders to a single IVIG infusion carried the MIF -173*C allele more frequently than responders (6/8=75% *vs*. 17/61=28%, OR 5.1, 95% CI 1.42-22.31 *p*<0.014).

In univariate analysis, CAA resulted significantly related with the presence of MIF -173*C allele (r_s=0.36, *p*<0.002) and with the response to IVIG infusion (r_s=0.32, *p*<0.007). After multiple regression analysis, weighted for response to IVIG, the effect of the number of received IVIG infusions disappeared, thus MIF -173*C allele resulted the single predictor of CAA and KD patients carrying a MIF -173*C allele were found to have an increased risk of coronary involvement (OR 7.7, 95% CI 1.36-16.1, *p*=0.021).

Discussion

Recently, MIF has gained interest for its role in the immunopathogenesis of several immune-mediated and inflammatory diseases, including KD (5). We showed that children carrying the MIF polymorphism -173*C had a higher percentage of CAA. In the same line, we found an association between the same polymorphism -173*C and a higher percentage of resistance to IVIG infusion, confirming the susceptibility to more severe disease.

MIF is present in and produced by endothelial cells, vascular smooth muscle cells, lymphocytes and macrophages, with a possible role in the progression of atherosclerosis (16, 17). A recent study has demonstrated that MIF serum levels are higher during the acute phase of KD when compared with patients in the subacute phase and with healthy controls, and related to IL-6 levels, highlighting its possible role in the disease pathogenesis (5). Our study is retrospective, and we did not have the possibility to measure MIF serum levels; however, it is worth noting that it has been shown that the serum concentration of MIF protein was higher in subjects carrying MIF -173*C in juvenile arthritis, consisting with the higher *in vivo* production of MIF by MIF -173*C carriers (7, 10).

Chen *et al.* provided evidence that neutralizing MIF bioactivity after experimental angioplasty in atherosclerosis-susceptible mice reduces vascular inflammation, cellular proliferation, and neointimal thickening in atherosclerosis-susceptible mice, supporting its role in modulating the biological response to vascular injury (18). These data prompted authors to suggest that MIF protein may be involved in the biological mechanisms that control endothelial injury, cellular proliferation and intimal thickening because of its anti-inflammatory, antiproliferative, and pro-apoptotic actions (18). Certainly, other polymophisms have already been shown to be related to the susceptibililty to KD and CAA complications, such as the polymorphism of the promoter region of IL-10 gene (19), of the receptor-ligand pair CCR5 and CCL3L1 (20), and more recently of ITPKC (21).

It has been shown that MIF, via different protein tyrosine kinases, is a potent angiogenic factor and has direct effects in up-regulating vascular and intercellular adhesion molecules in human peripheral blood monocytes (22). Since in inflammatory diseases adhesion of inflammatory cells to vascular endothelium is a critical step in leukocyte recruitment, a MIF-mediated proinflammatory effect on monocytes recruitment has also been suggested (23).

Carrying the -173*C MIF polymorphism could therefore be a risk factor for children with KD to a higher susceptibility to vascular injury, via different degree of regulation in these MIF-mediated monocytes/endothelial reciprocal interactions. As previously suggested for others diseases such as rheumatoid arthritis and atherosclerosis (23), MIF and its signalling pathways might be potential targets also in KD treatment .

The high percentage of CAA observed in KD children carrying this allele, as well as their IVIG treatment resistance, are consistent with previous observations and, particularly, with the poorer outcome of a inflammatory disease such as systemic JIA (10, 11). In line with what already hypothesised (10) in other systemic inflammatory diseases, our observations seem to suggest that the potential association of MIF -173*C allele and CAA might be secondary to an increased production of MIF overriding the host-defence mechanisms on immune and inflammatory responses. In this scenario, specific MIF inhibition could also be considered a potential therapeutic option for damping inflammation in KD and reducing CAA incidence, particularly in children with MIF -173*C.

In conclusion, our study suggests a potential relationship between a MIF polymorphism and risk of CAA in KD; to our knowledge this finding has not been reported before. Further studies with concomitant protein determination assay in different stages of disease and in a larger cohort will be necessary to clarify this potential link.

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