MMP-9 microsatellite polymorphism and susceptibility to exudative form of age-related macular degeneration

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Purpose: To assess if a polymorphism (PM) of the microsatellite (CA_{13-27}) in the promoter region of Matrix Metalloproteinase 9 (*MMP-9*) was associated with the exudative form of age-related macular degeneration (AMD) and to its risk factors. **Methods:** In 107 patients with AMD (AMD Group) and 223 age- and gender-matched controls (Control Group) with cataract, demographic, clinical data, and *MMP-9* PM have been compared. **Results:** The comparison of allelic frequencies showed a different pattern of CA repeats between AMD and Control Group ($P < 0.00005$), in particular the prevalence of longer microsatellites ($\geq$ 22 CA repeats) was higher in AMD than in Control Group (O.R. 2.49, 95% CI 1.71 - 3.37, $P < 0.001$). Analyses of genetic frequencies gave similar results. Logistic regression confirmed that 22 or more CA repeats are associated to AMD. The only association between MMP-9 PM and other risk factors for AMD was with BMI (Spearman's $R = 0.298$, $P < 0.00005$): all patients with both microsatellites $\geq$ 22 CA repeats were overweight or obese (χ^2 test $P < 0.0005$, compared to other genotypes). **Conclusions:** Longer microsatellites in the promoter of MMP-9 are associated to the exudative form of AMD and to body mass index, a well-known risk factor for the disease. ***Genet Med* 2005:7(4):272–277.**

Key Words: *MMP-9, age-related macular degeneration, choroidal neovascularization, weight, body mass index*

Age-related macular degeneration (AMD) is the leading cause of vision loss in the industrialized countries.[1,2] Early AMD is characterized by focal or diffuse deposition of extracellular material (collagen, lipid, and phospholipid) between retinal pigment epithelium (RPE) and Bruch's membrane, forming drusen or basal laminar deposits, respectively.[3–5] This process is associated with progressive degeneration of RPE cells and photoreceptors.[5–7] Advanced AMD is characterized by severe visual loss associated with geographic atrophy and/or choroidal neovascularization (CNV).

Although some risk factors such as age, gender, race, genetic influence, smoking, hypertension, cardiovascular diseases, body mass index (BMI), or hypercholesterolemia have been identified, the etiopathogenesis of AMD is still unclear.

Pathophysiology of AMD implicates extracellular matrix modification: some metalloproteases, particularly MMP-9, are increased in the retina of patients with exudative AMD, suggesting a potential role in the development of CNV.[8–10]

MMP-9 transcriptional activity is regulated by genetic polymorphisms (PMs) of the promoter region.[11] One of them consists of a sequence of CA (Cytosine-Adenine) repeats around the −90 region. It has been postulated that this microsatellite, allowing the DNA to switch to a Z structure, eases the opening of the double strand of DNA and its transcription. In in vitro studies, a different number of CA repeats seems to control the expression of the gene, i.e., 14 repeats compared to 21 repeats account for 40% reduced expression.[12] In general population, however, the number of CA repeats per each allele ranges from 13 to 27 with two modes at 14 and 21 repeats.[11] Little is still known about *MMP-9* expression in carriers of 22 or more CA repeats, which account for about one third of the general population.[13,14]

Data obtained in mice (having a *MMP-9* promoter quite similar to the human) indicate that mesangial cells carrying 24 CA repeats have a more than 20 times increased MMP-9 expression when compared to the same cells of a strain with 20 repeats.[15] Therefore, one allele with 24 repeats should express more protein than two alleles with 20.

The aim of this study was to evaluate the role of this PM in susceptibility of the exudative form of AMD assessing an association between AMD and higher number of repeats within MMP-9 promoter. In the present study, we compared the microsatellite polymorphism of the promoter region of *MMP-9* gene in AMD patients and in age- and gender-matched con-

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trols chosen among patients admitted for cataract surgery. Moreover, because the link between some risk factors and AMD is sometimes speculative, we analyzed if risk factors for AMD could be associated to this polymorphism.

METHODS

A consecutive series of patients affected by exudative AMD, referring to the angiographic center of the Eye Clinic of the University of Trieste between January 2002 and January 2003, were considered. Inclusion criteria were age older than 50 years and diagnosis of exudative AMD with predominantly classic subfoveal CNV. Exclusion criteria were the presence of any other cause of CNV, such as pathologic myopia, angioid streaks, presumed ocular histoplasmosis, chorioretinal inflammatory diseases, trauma, hereditary retinal disorders, or idiopathic CNV. Eyes presenting polypoidal CNV and retinal vascular anomalous complex were also excluded.

Diagnosis of exudative AMD was based upon the presence of the following: visual acuity $<$ 20/20 with distortion on Amsler grid testing; drusen in both eyes; presence of sensory retina detachment, and/or retinal hemorrhages, and/or exudates; or detection of subfoveal CNV on fluorescein angiography. Eligible patients underwent a complete ophthalmologic assessment including history, with collection of the number of relapses and of the disease duration, fluorescein angiography, and best-corrected visual acuity evaluation on ETDRS charts.

The control group (Control Group) consisted of age- and gender-matched patients referring for cataract surgery at the Eye Clinic of the University of Trieste during the same time period. Risk factors evaluated were diabetes (fasting blood sugar $>$ 116 mg/dL), dyslipidemia (total cholesterol $>$ 200 mg/dL or HDL-cholesterol $<$ 40 mg/dL in women or $<$ 35 mg/dL in men), hypertension (systolic and/or diastolic pressure $>$ 140/85 mm Hg on more measurements), and smoking habit (considered as a smoker a patient regularly smoking more than 3 cigarettes a day). Control and AMD patients were also similar for ethnic origin and city of residence; all gave their written informed consent to the study, approved by the local Ethical Committee. The study adhered to the tenets of the declaration of Helsinki.

MMP-9 polymorphism

Blood samples from patients and controls were collected at hospital admission. DNA was extracted from peripheral leukocytes with the Promega Wizard genomic DNA purification kit. The details of the method for the determination of *MMP-9* PM (PCR amplification and capillary electrophoresis analysis) have been previously published.[16]

Power calculations and statistical analysis

The study has been sized to detect a difference in the prevalence of grouped alleles of 12% or more with a ratio of 1 patient/2 controls and $\alpha = 0.05$ and $1-\beta = 0.80$. These assumptions required at least 198 and 395 alleles, respectively. The final figures of alleles studied originated from genotyping procedure in batches: all patients genotyped have been included for additional confidence.

The allele frequencies of *MMP-9* polymorphism between AMD and Control Group were compared with the χ^2 test. Moreover, in order to find a cutoff, i.e., the interval of repeats that could best discriminate the two groups, multiple χ^2 test, with odds ratio (OR) calculations, were performed. The dichotomizations consisted in dividing alleles into two clusters, according to the number of repeats (e.g., 13 vs. $\geq$ 14 repeats, then $\leq$ 14 vs. $\geq$ 15, and so on); and then perform repeated χ^2 and OR tests. Genotype frequencies have been compared with ORs with an analogous procedure, but considering only the longest microsatellite. For repeated comparisons, the usual sig-

Table 1
Primers sequence and PCR settings for MMP-9 polymorphism analysis

Forward Primer	GACTTGGCAGTGGAGACTGCGGGCA	
Reverse Primer	GACCCCACCCCTCCTTGACAGGCAA	
Amplified sequence	$-204 \rightarrow -48$	
	T°	Time (sec)
Pre-denaturation	94	300
Denaturation	94	60
Annealing	69	60
Extension	72	60
Number of cycles	35	
Final Extension	72	600

Table 2
General data in AMD Group and Control Group

	AMD	Controls	P
Number	107	223	
Men %	30.8	32.9	
Men/women	33/74	73/179	
Age, y	71 ± 11	75 ± 10	
Height, cm	162 ± 9	164 ± 11	
Weight, Kg	71 ± 14	70 ± 15	
BMI	26.9 ± 4.7	26.2 ± 6.9	
Systolic BP, mm Hg	142 ± 15	139 ± 23	0.000
Diastolic BP, mm Hg	82 ± 8	77 ± 10	0.001
Diabetes n (%)	10 (9.3)	28 (13.1)	
Dyslipidemia n (%)	15 (14)	22 (10)	
Smoking habits n (%)	10 (9.3)	14 (12.1)	
Thyroid dysfunction n (%)	8 (7.5)	2 (1.8)	0.041
Previous vascular events n (%)	16 (15)	44 (38)	$<$0.0005

Diabetes was diagnosed when fasting blood sugar was $>$ 116 mg/dL, dyslipidemia if total cholesterol $>$ 200 mg/dL or HDL-cholesterol $<$ 40 in women or $<$ 35 mg/dL in men, hypertension when systolic and/or diastolic pressure were $>$ 140/85 mm Hg on more measurements, and smoking habit considering as a smoker a patient or control regularly smoking more than 3 cigarettes a day.

nificance *P* level has been corrected, according to Bonferroni, and the (two-tailed) *P* values < 0.004 were considered statistically significant.

For comparisons between groups and assessment of the relationship between risk factors and *MMP-9* PM, parametric and nonparametric statistics were used as appropriate, and (two-tailed) *P* value < 0.05 was considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Version 11.0, SPSS Inc, Chicago, IL).

RESULTS

AMD Group consisted of 107 patients, and Control Group of 222 age- and gender-matched patients referring for cataract surgery. All were White. Systolic and diastolic blood pressure were higher in AMD patients. In the past history, Control Group patients had had more previous vascular events (angina, acute myocardial infarction, TIA, and stroke). A trend toward a lower stature ($P = 0.056$) and higher BMI ($P = 0.066$) was detected in AMD Group (Table 2).

Allelic and genotype frequencies

All genotyping were successful. Control and AMD groups showed a statistically different allele frequency of microsatellite PM (Table 3) with the pattern of the latter group skewed toward the right ($P < 0.0005$) (Fig. 1 and Table 3). Multiple ORs, after Bonferroni correction, showed that alleles with 22 to 27 repeats were associated to AMD (Fig. 1). Alleles with 22 or more repeats were more frequent in AMD Group (OR = 2.48, 95%CI 1.71–3.37; $P < 0.0005$). The analysis of genetic frequency of AMD and Control groups, considering only the longest allele in each patient or control, confirmed the evidences obtained from allelic frequency: carriers of a microsatellite with ≥ 22 repeats had an OR of 3.00 (95% CI 1.86–4.83; $P < 0.0005$; Table 4) of having AMD. The dichotomization between 20 and 21 ($P = 0.011$), was nonsignificant considering

Table 3
Genetic frequencies in Controls and ADM patients

No. of CA repeats	AMD	Controls	χ^2P[a]	OR	95% CI
13		16 (3.6)			
			0.005		
14	122 (57)	268 (60.1)			
			0.099		
15	5 (2.3)	12 (2.7)			
			0.078		
16	2 (0.9)	2 (0.45)			
			0.100		
18	0 (0.0)	3 (0.67)			
			0.069		
19	0 (0.0)	4 (0.9)			
			0.040	1.42	1.01–2.00
20	5 (2.3)	23 (5.2)			
			0.004	1.66	1.2–2.35
21	4 (1.9)	37 (8.3)			
			0.000	2.48	1.71–3.37
22	36 (16.8)	54 (12.1)			
			0.000	3.57	2.12–5.99
23	17 (7.9)	14 (3.1)			
			0.000	4.02	1.99–8.05
24	20 (9.3)	11 (2.5)			
			0.186	3.15	0.52–18.87
25	3 (1.4)	2 (0.45)			
	214 (100)	446 (100)			

χ^2, OR (95% CI) for each possible dichotomization are reported.
[a]Significance level after Bonferroni correction < 0.005. Numbers in parenthesis are the percentage within the group.

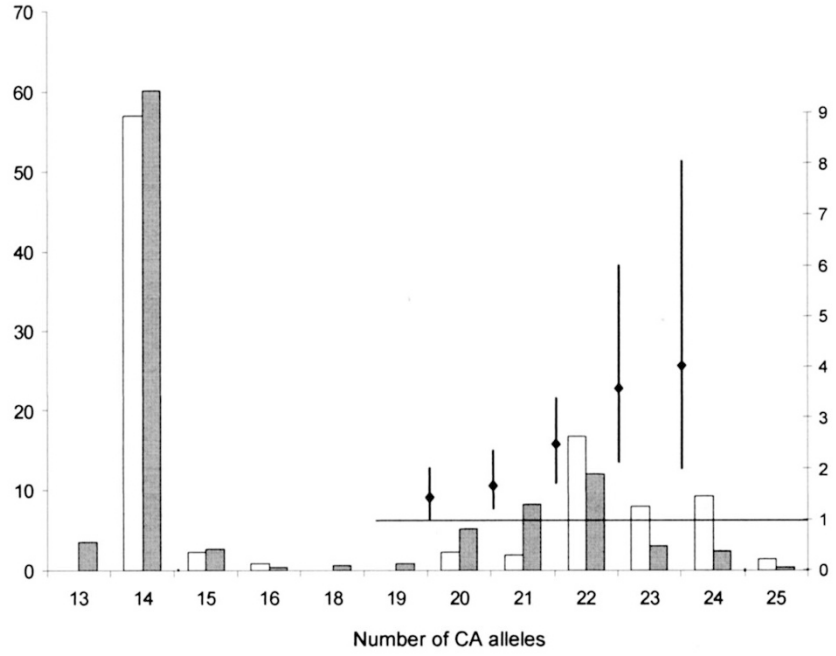

Fig. 1. Comparisons of allele frequencies in the populations of AMD Group and Control Group. Histograms (empty for AMD patients and gray for cataract patients) represent the allelic frequency (left axis). On the right axis are shown the ORs for association of the different grouping of alleles with AMD. Repeated dichotomizations of alleles of patients and controls have been performed according to the number of repeats (e.g., 13 and $\geq$ 14 repeats, then $\leq$ 14 and $\geq$ 15, and so on). ORs and 95% CI for association of a certain number of repeats with AMD were calculated and reported in figure as "high and low" vertical bars.

the Bonferroni correction. Logistic regression analysis showed that, compared with risk factors for AMD (gender, age, diabetes, hypertension, smoking habit, BMI, and dyslipidemia), the status of carrier of a microsatellite $\geq$ 22 repeats was the only variable entering into the equation ($P = 0.011$). When *MMP-9* PM was removed from the variable list, the most important variable became BMI ($P = 0.019$).

MMP-9 PM did not seem to influence the clinical course of AMD: the number of relapses in AMD patients carrying $\geq$ 22 repeats was similar to that of the other AMD patients (2.3 ± 1.3 vs. 2.3 ± 1.4; P = NS), even after stratification by observation time. Moreover, a Kaplan Maier curve did not show any difference in the time course of the relapses (data not shown).

Association between risk factors and *MMP-9* PM

Patients and controls were studied. No differences were found between the main factors for AMD (gender, age, diabetes, hypertension, smoking habit, and dyslipidemia) and *MMP-9* PM. The only association with a risk factor detected was a strong linear correlation between number of repeats in the longer microsatellite and weight and, particularly, with BMI (Pearson's $R = 0.251$, Spearman's 2.98; $P < 0.00005$ for both). Patients (Table 5) carrying both alleles $\geq$ 22 repeats showed higher BMI and weight when compared to the other patients. All patients with both microsatellites $\geq$ 22 CA repeats were overweight (BMI range 27.6–47.6) and 50% were obese (median 29.78).

DISCUSSION

A methodological issue had to be addressed in our study design: AMD shares many risk factors with atherosclerosis, implying also a reduced survival of the patients.[17] The relationship between AMD and atherosclerosis is not completely understood. To assess if this PM is associated to AMD and not only to atherosclerosis, the Control Group had to resemble AMD in the atherosclerotic risk profile. Cataract patients too showed a reduced survival[18] and, therefore, the comparison seemed suitable. Recently, a study from Borger et al.[19] has confirmed that the risk profile and mortality in the two diseases were similar. For these reasons, cataract patients rather than random healthy population have been chosen for this study.

The development of CNV is the major cause of severe visual loss in exudative AMD. Little is known regarding the molecular mechanism underlying the choroidal angiogenesis; several factors have been implicated in the development of CNV including VEGF, TGF-β, FGF, TNF, Placental GF, PEDF. Overall, a combination of the effects of proangiogenic and antiangiogenic growth factors, in association with a localized damage to Bruch's membrane, are the necessary conditions to the choroidal angiogenesis and its expansion into the subretinal space.

MMPs are a family of structurally related enzymes, capable of degrading most of the component of the extracellular matrix, which may play an important role in the extracellular matrix remodeling during angiogenesis. Particular interest has been focused on MMP-9 due to its ability to degrade basement membrane components such as type IV collagen.

Table 4
Allelic frequencies in ADM patients and Controls (Patients/Controls)

13	14	15	16	18	19	20	21	22	23	24	25	
0/5						0/3	0/3					13
	35/90	3/6	2/0	0/3		3/10	1/17	19/36	12/10	10/5	2/2	14
			0/2				0/1	1/3		1/0		15
												16
					0/2							18
					0/2							19
							0/1	1/3		1/2		20
							0/5	3/2				21
								1/2	4/4	5/4	1/0	22
										1/0		23
										1/0		24
							0.011	<0.0005	<0.0005	<0.0005		P
							1.83	3.00	4.0	4.18		OR
							1.15–2.92	1.86–4.83	2.27–7.04	2.01–8.69		95% CI

Significance level <0.0044.

Table 5
Anthropometric data and CA_n repeats

	Both alleles ≤ 21 CA repeats (n = 107)	One allele ≤ 21 and one ≥ 22 CA repeats (n = 95)	Both alleles ≥ 22 CA repeats (n = 21)	P
Height, cm	165 (160–170)	161 (155–169)	162 (155–170)	0.270
Weight, kg	66 (59–78)	68 (61.5–77.5)	78 (74–93)	0.016
BMI	25.7 (23.2–28.4)	25.3 (22.9–29.2)	29.7 (28–34.1)	0.004
Normal weight n (%)	57 (53.3)	39 (41.0)	0 (0)	
Overweight n (%)	42 (39.2)	43 (45.3)	11 (52.4)	<0.0005
Obese n (%)	8 (7.5)	13 (13.7)	10 (47.6)	

Statistical analysis for height, weight, and BMI was performed by Kruskal Wallis test and for Normal weight (BMI < 25), Overweight (BMI ≥ 25 and < 30), and Obese (BMI ≥ 30) by χ^2 test.

Analysis of surgically removed subfoveal fibrovascular membranes from patients affected by AMD showed MMP-9 expression at the margins of the membrane and in proximity of a thickened Bruch's membrane layer under the retinal pigment epithelial cells.[20]

Studies using gelatin substrate zymography on homogenates of Bruch's membrane and of human retina choroid demonstrated the presence of MMP-9 and MMP-2 in Bruch's membrane and in the choroids; moreover, both levels of MMP-2 and MMP-9 tended to increase with aging.[21]

Berglin[22] and Lambert[9] have described a significant reduction in the development of laser-induced CNV in MMP-2 and -9 knockout mice although, surprisingly, the association of this microsatellite has not been detected in high throughput genome analysis.[23]

In the present study, longer microsatellites in the promoter of MMP-9 are associated with CNV development in patients with AMD. These data are also confirmed by logistic regression analysis. The main result is that carriers of one allele with ≥ 22 repeats have a more than doubled risk of being an AMD patient. A similar OR has also been reported for a well-identified risk factor like detection of five or more drusen.[24] This is, to our knowledge, the first time that an association between this PM and AMD is demonstrated. A post hoc analysis showed that the current differences in allelic and genetic frequencies for ≥ 22 repeats observed in AMD patients and cataract controls are supported with an α value of 0.01 and power of 97%. MMP-9 has been involved in many other diseases, such as cancer,[12] atherosclerosis,[25] rheumatoid arthritis,[26,27] vascular remodeling,[28] but the study of its polymorphisms have not been con-

clusive. The approach we followed has already been studied on multiple sclerosis with results consistent with the present study: the very same alleles, i.e., those with ≥ 22 repeats, are associated to the susceptibility of multiple sclerosis.[16]

This polymorphism does not account for the disease but, facilitating MMP-9 expression, might act in increasing the vascular permeability of the vessels or the neovascularization, as seen in vitro and in other experimental models.

The clinical relevance of these findings lies in the possibility to predict the susceptibility of the disease and suggests that inhibition of MMP-9 expression or activity might retard the progression of AMD.

High BMI value is associated to higher risk for AMD[29,30] and, independent from AMD, to *MMP-9* PM. The role of MMP-9 in adipose tissue maturation has been previously documented in animal models[31] and, in particular, of preadipocytes[32]; conversely, MMP-9 inhibitors impair adipose tissue development in mice.[33] Although intriguing, these results require further research before we understand the relationship among AMD, BMI, and *MMP-9* PM.

The study has not established a causal relationship of *MMP-9* PM with AMD or one of its clinical features, although it offers a demonstration on the clinical association of *MMP-9* PM with wet AMD. Several candidate genes have been studied in the last years, but not all have had an extensive evaluation. More studies are needed before we can understand the role of mutations and PM in development of AMD.

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