A polymorphism of HMGA1 protects against proliferative diabetic retinopathy by impairing HMGA1-induced VEGFA expression

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Diabetic retinopathy (DR) is a major complication of diabetes mellitus, and is the leading cause of blindness in working-age people. Usually, DR progresses from the asymptomatic non-proliferative DR that does not significantly alter vision, to proliferative DR (PDR), which can result in aberrant retinal neovessel formation and blindness. The High-Mobility-Group A1 (HMGA1) protein is a transcriptional master regulator of numerous genes, including metabolic and inflammatory genes, which, by modulating the expression of angiogenic factors, may induce retinal neovascularization, a hallmark of PDR. Herein, we examined the relationship between HMGA1 rs139876191 variant and DR. Results revealed that patients with type 2 diabetes, who were carriers of the HMGA1 rs139876191 variant had a significantly lower risk of developing PDR, compared to non-carrier diabetic patients. From a mechanistic point of view, our findings indicated that, by adversely affecting HMGA1 protein expression and function, the HMGA1 rs139876191 variant played a key role in this protective mechanism by downregulating the expression of vascular endothelial growth factor A (VEGFA), a major activator of neovascularization in DR. These data provide new insights into the pathogenesis and progression of DR, and may offer opportunities for discovering novel biomarkers and therapeutic targets for diagnosis, prevention and treatment of PDR.

Diabetic retinopathy (DR) is the most common microangiopathic complication of diabetes mellitus, affecting over 30% of diabetic patients, and the leading cause of blindness among working-age adults in developed countries1,2. With the widespread diffusion of type 2 diabetes, the prevalence of DR is increasing worldwide along with rising health-care expenses and labor costs3. Thus, developing strategies to prevent and effectively treat DR is extremely important. Conceptually, DR consists of an early non-proliferative stage (NPDR) characterized by microaneurysms, dot and blot hemorrhages, retinal vascular leakage with exudate accumulation, and a more advanced, proliferative stage (PDR), in which visual loss can occur from either proliferation of new retinal vessels, or increased permeability of retinal blood vessels3. Several lines of evidence indicate that both increased vascular permeability and neovascularization in PDR may depend on the local production of angiogenic factors, inflammatory cytokines, chemokines and growth factors, in addition to components of the extracellular matrix, which will be substrates for endothelial migration4. In this context, the vascular endothelial growth factor-A (VEGFA), a major activator for angiogenesis5, is believed to play significant roles by inducing neovascularization and increasing permeability of retinal vessels4. In line with this, VEGFA expression is induced by hyperglycemia and hypoxia4, two hallmarks of diabetic complications, whereas levels of VEGFA are markedly increased in the

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vitreous of diabetic patients with active PDR. However, despite many investigations, the underlying etiology of DR is largely unknown, although genetic factors may contribute to both the occurrence and severity of this disease. In this regard, the heritability estimates reported seem to be as high as 27% for NPDR and 50% for PDR.

Investigations using different approaches, including the more recent genome-wide association studies (GWAS), have been conducted, but success in identifying the genetic variants involved in DR has been limited.

HMGA1 is an architectural transcription factor that acts as a dynamic regulator of chromatin structure and gene activation. Defects in HMGA1 gene and protein expression have been associated with insulin resistance and increased susceptibility to type 2 diabetes in humans and mice, whereas protection against insulin resistance has been reported in transgenic mice overexpressing HMGA1. Further evidence, implicating the HMGA1 locus as one conferring high risk for the development of type 2 diabetes, has been provided recently by showing that a specific single-nucleotide insertion at position 13 of exon 6 of the HMGA1 gene (IVS5–13insC; more precisely, c.136–14_136–13insC; rs139876191), significantly associates with type 2 diabetes in a transthetic meta-analysis. Also, HMGA1 is a hypoxia-inducible factor that modulates the expression of several angiogenic proteins, other than the production of cytokines, chemokines and adhesion molecules that, by triggering endothelial dysfunction via inflammation, may play a pathogenetic role in DR. Moreover, HMGA1 is highly expressed in murine retinas, while cis-regulatory elements for HMGA1 are more abundant in promoters of genes preferentially expressed in retinal endothelial cells.

Based on the above considerations, it was the aim of this study to evaluate the association of the HMGA1 gene polymorphism rs139876191 with DR, and determine the role of this allele’s variant, if any, in the pathogenesis of this diabetic complication.

Results

Demographic, anthropometric, clinical and biochemical features of enrolled patients are in Table 1. As assessed by non-parametric Mann-Whitney test, diabetic patients with PDR differed from diabetic control patients without DR in the following respects: higher HbA1c (P = 0.002), FPG (P = 0.004), systolic and diastolic BP (P < 0.001) and a higher prevalence of nephropathy (P = 0.015) (Table 1). In addition, patients in the PDR and NPDR groups had a significantly lower prevalence of hypoglycemic drug use (P < 0.001 and P = 0.006, respectively), a higher insulin use (P < 0.001 and P = 0.005, respectively) and a higher hypolipidemic drug treatment (P < 0.001 in both groups), as compared with diabetic control individuals without DR (Table 1).

Case-control association of HMGA1 rs139876191. The HMGA1 rs139876191 was less common in diabetic patients with PDR than in those without PDR. Among the 436 patients with PDR, 20 (4.6%) were heterozygous carriers (MAF 4.01%), and none was homozygous (Table 2). Similarly, among 936 diabetic control subjects without DR, 75 (8.0%) were heterozygous and 1 (0.2%) was homozygous for the rs139876191 polymorphism (MAF 2.52%) (Table 2). In contrast, the 587 diabetic patients with NPDR, 50 (8.5%) were heterozygous carriers (MAF 4.26%) and no subject was homozygous (Table 2). Similarly, among 936 diabetic control subjects without DR, 75 (8.0%) were heterozygous carriers (MAF 4.01%), and none was homozygous (Table 2). No significant deviation from Hardy-Weinberg equilibrium was detected (P = 0.16 for PDR cases; P = 0.28 for NPDR cases; P = 0.20 for controls). Logistic regression analysis with age and gender as covariates indicated that the presence of HMGA1 rs139876191 was associated with over 40% [OR 0.573 (95% CI, 0.348–0.943), P = 0.029] lower risk of PDR (Table 2). The same relationship [OR 0.518 (95% CI, 0.309–0.868), P = 0.013] was observed when other covariates (i.e., duration of diabetes, hypertension, HbA1c, HDL-cholesterol, hypolipidemic and antihypertensive therapy) were included in the analysis (Table 2). In contrast, no association of the rs139876191 variant was observed with NPDR (Table 2), thus supporting the specific effect of this variant on PDR. It was interesting to note that during the study period, 38 diabetic patients initially classified as NPDR developed PDR, but only two of them were carriers of the rs139876191 variant.

Association of the HMGA1 rs139876191 with clinical and biochemical features. To determine whether the HMGA1 rs139876191 was also associated with distinct clinical and biochemical characteristics, several quantitative and qualitative variables were evaluated in both carrier and non-carrier groups using multiple regression analysis (Table 3). Age, age at diagnosis of diabetes and BMI were similar in the two diabetic patient groups, and no significant differences were detectable among groups with regard to hypertension prevalence. No significant differences were also observed between these two groups concerning FPG levels, HbA1c, serum cholesterol (total, HDL-, LDL-cholesterol) and triglycerides.

Functional analysis of the HMGA1 rs139876191. The influence of the rs139876191 variant on the functionality of HMGA1 gene was analyzed using the minigene strategy, an approach that has the potential to evaluate the significance of intronic polymorphisms. Using this strategy, we analyzed the impact of the rs139876191 polymorphism on the functionality of the HMGA1 mRNA and protein levels, as this was of crucial importance for evaluating the relevance of this mutation on gene expression. For these experiments, either wild-type or mutant minigenes were transfected in HEK-293 cells and endogenous HMGA1 mRNA was measured by qRT-PCR. As shown in Fig. 1a, HMGA1 mRNA was significantly reduced in cells transfected with the mutant minigene construct compared to cells transfected with the wild-type minigene, thus indicating that the rs139876191 variant is functional and exhibits a dominant negative effect over endogenous HMGA1. As shown in time-course experiments, the negative effect of the mutant minigene on endogenous HMGA1 mRNA expression was time-dependent, with maximal inhibition at 72h post transfection (Fig. 1b). Interestingly, the magnitude of endogenous HMGA1 protein and mRNA reductions in HEK-293 cells transfected with the mutant HMGA1 minigene was consistent with the reduction of HMGA1 expression previously observed in blood monocytes of diabetic patients with the rs139876191 variant.

Influence of the HMGA1 rs139876191 on serum cytokines and other serum factors. The role of inflammatory proteins, cytokines and adhesion molecules in the onset and progression of DR is well known. As
negative effect of the rs139876191 variant over endogenous HMGA1 expression. Patients, and this is consistent with the above experimental results in vitro polymorphism can be ascribed, at least in part, to the lower concentration of VEGFA serum levels seen in such carriers. It can be hypothesized that the reduced risk of developing PDR in type 2 diabetic patients carrying the rs139876191 allele is associated with decreased VEGFA concentration, which may be explained by the polymorphism in the HMGA1 promoter region. A further association of this variation with the lower risk of developing PDR in type II diabetic patients is supported by the results of the present study. In fact, compared to control subjects, carriers of the rs139876191 variant showed a significant reduction in the levels of VEGFA serum in type 2 diabetic patients, indicating a potential role of this polymorphism in modulating the VEGFA expression, which is a key regulator of retinal angiogenesis.

Table 1. Clinical and biochemical baseline characteristics of enrolled patients. Data are medians (IQR) or n (%). Non-parametric Mann-Whitney test was used for distribution comparisons of quantitative variables. The two-tailed Fisher Exact Test was used for proportion comparisons between groups. Values versus control PDR < 0.05. BMI, body mass index; BP, blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol. *Includes myocardial infarction, coronary heart disease, and stroke. **Refers to people with urine albumin:creatinine ratio > 30 μg/mg, or with albumin excretion rate > 30 mg/day (ADA criteria). ☑Includes foot ulceration, lower-extremity amputation, and sensory impairment.

| Control n = 936 | NPDR n = 587 | PDR n = 436 |
|----------------|-------------|------------|
| Ethnicity      | Caucasian   | Caucasian  | Caucasian |
| Female (%)     | 505 (54.0)  | 282 (48.0) [0.027] | 212 (48.6) [0.072] |
| Age (yr)       | 66 (61–75)  | 66 (61–72) [0.926] | 67 (60–75) [0.374] |
| Age at onset of diabetes (yr) | 52 (46–58) | 51 (47–58) [0.748] | 51 (46–58) [0.499] |
| Duration of diabetes (yr) | 13 (11–18) | 14 (11.5–17) [0.476] | 15 (11–18) [0.607] |
| Family history of diabetes | 582 (62.2) | 374 (63.7) [0.550] | 280 (64.2) [0.472] |
| BMI (Kg/m²)    | 27.9 (26.1–30.2) | 27.8 (26.0–30.5) [0.727] | 27.4 (26.0–30.0) [0.210] |
| Systolic BP (mmHg) | 130 (125–140) | 135 (125–140) [<0.001] | 135 (125–145) [<0.001] |
| Diastolic BP (mmHg) | 80 (70–80) | 80 (75–80) [0.053] | 80 (75–80) [<0.001] |
| Hypertension (140/90) | 499 (53.3) | 328 (55.9) [0.342] | 280 (64.2) [0.001] |
| Antihypertensive therapy (n) | 325 (34.7) | 218 (37.1) [0.350] | 178 (40.8) [0.030] |
| FPG (mg/dL)    | 158 (150–176) | 159 (150–180) [0.984] | 165 (150–181) [0.004] |
| HbA1c (%)      | 7.4 (7.2–8.2) | 7.6 (7.3–8.4) [0.755] | 7.8 (7.3–8.5) [0.002] |
| HbA1c (mmol/mol) | 60 (55–66) | 60 (56–68) | 62 (56–69) |
| Diet treatment alone (n) | 3 (0.3) | 1 (0.2) [0.099] | 2 (0.5) [0.656] |
| Non-insulin hypoglycemic agents only (n) | 527 (56.3) | 288 (49.1) [0.006] | 131 (30.0) [<0.001] |
| Insulin therapy (n) | 406 (43.3) | 298 (50.8) [0.005] | 303 (69.5) [<0.001] |
| Total cholesterol (mg/dL) | 1690 (1510–1900) | 1690 (1490–1900) [0.937] | 1690 (1493–1920) [0.942] |
| HDL-C (mg/dL)  | 47.0 (43.0–52.0) | 46.0 (40.0–55.0) [0.116] | 46.0 (40.0–54.0) [0.052] |
| LDL-C (mg/dL)  | 94.2 (76.4–114.6) | 98.0 (76.1–113.6) [0.982] | 94.8 (78.2–114.9) [0.969] |
| Triglycerides (mg/dL) | 125 (99–158) | 125 (99–158) [0.613] | 123 (95–160) [0.300] |
| Hypolipidemic therapy (%) | 405 (43.3) | 329 (56.0) [<0.001] | 272 (64.2) [<0.001] |
| Creatinine (mg/dL) | 1.1 (1.0–1.2) | 1.0 (0.9–1.2) [0.127] | 1.1 (0.9–1.2) [0.094] |
| Macroangiopathy (n)* | 216 (23.1) | 144 (24.5) [0.536] | 114 (26.1) [0.223] |
| Nephropathy (n)** | 105 (11.2) | 79 (13.4) [0.197] | 70 (16.1) [0.015] |
| Foot disease (n)** | 195 (20.8) | 134 (22.8) [0.338] | 111 (25.5) [0.052] |

they may serve as potentially useful biomarkers for early detection and prognosis of DR, numerous studies have been conducted over the past years analyzing serum levels of these molecules in affected diabetic patients. However, conflicting data have been published on this subject, mostly because of the many confounding factors, such as comorbid disorders, variability in glucose levels and drug intake, which may affect analytical results, thereby reducing the clinical significance of changes in serum levels of these markers. Because of these considerations and in order to explore the influence on serum cytokines, growth factors and adhesion molecules exerted by the HMGA1 rs139876191 variant, we selected 37 carriers of the rs139876191 variant and 97 non-carriers, carefully matched for age, sex, BMI and FPG. As shown in Table 4, fasting serum VEGFA levels were significantly lower in healthy subjects compared to wild-type subjects (P = 0.019). No other associations were statistically significant, although we cannot exclude a type II error due to the small number of analyzed subjects (Table 4). Based on this association and on the established role of VEGFA in retinal neovascularization, it can be hypothesized that the reduced risk of developing PDR in type 2 diabetic patients carrying the rs139876191 polymorphism can be ascribed, at least in part, to the lower concentration of VEGFA serum levels seen in such patients, and this is consistent with the above experimental results in vitro with minigenes, showing a dominant negative effect of the rs139876191 variant over endogenous HMGA1 expression.

**HMGA1 regulates the expression of VEGFA.** The relationship between HMGA1 and VEGFA was further investigated in cell transfection studies using reporter gene assays and confirmed in vivo, in Hmgal1-knockout mice. As shown in Fig. 2a, transfection of HMGA1 expression vector significantly increased VEGFA luciferase activity in VEGFA-Luc-transfected HepG2 cells, and this effect occurred in a dose-dependent manner. Consistent with this, VEGFA mRNA levels were reduced in both HepG2 and HUVEC cells pretreated with siRNA targeting HMGA1 (Fig. 2b). These results were further supported by functional genomics data from ARPE-19 cells, a human retinal pigment epithelium cell line ideally suited for VEGF expression analysis. As shown in Fig. 2c,
VEGFA-Luc activity significantly increased in ARPE-19 cells overexpressing HMGA1, whereas VEGFA mRNA levels were decreased by siRNA targeting HMGA1, thus indicating that HMGA1 is required for proper transcription of the VEGFA gene. This conclusion was supported by the results obtained from Hmga1−/− mice, in which, in line with findings in humans, serum levels of VEGFA were significantly lower when compared with normal wild-type mice (Fig. 2d). To exclude any discrepancy between serum and retinal VEGFA, and to corroborate data in humans, VEGFA gene expression was also investigated in retinal tissue from Hmga1−/− mice. Consistent with the above results, VEGFA mRNA was significantly reduced in retina from mutant mice compared with wild-type controls (Fig. 2e).

HMGA1 and hypoxia. The promoting effect of hypoxia on VEGFA expression is well characterized and it may constitute a major trigger mechanism for VEGFA-induced neoangiogenesis in PDR. Therefore, we finally evaluated the role of HMGA1 on VEGFA expression in hypoxia. For this purpose, normoxic HepG2 cells were exposed for 24 h to CoCl2, a known chemical hypoxic mimetic, and VEGFA and HMGA1 mRNA levels were measured. As shown in Fig. 3a, VEGFA mRNA increased two-three fold in cells maintained in hypoxic conditions, and this increase paralleled the increase of HMGA1 mRNA abundance. However, when Hmga1-knockdown HepG2 cells were exposed to CoCl2, hypoxia-induced VEGFA and HMGA1 expressions were significantly blunted (Fig. 3a). These data were corroborated by ChIP-qPCR assays, showing that binding...
of HMGA1 to the endogenous VEGFA locus was considerably decreased in whole, intact HepG2 and ARPE-19 cells exposed to HMGA1 siRNA, either in normoxia or hypoxia (Fig. 3b), thus confirming the essential role of HMGA1 in VEGFA expression, and supporting the notion that a deficit of HMGA1 as that observed in type 2 diabetic patients with the HMGA1 rs139876191 may protect against hypoxia-induced damages, including PDR, and perhaps other VEGFA-related diabetic complications.

Discussion

Herein, we evaluated the association of the HMGA1 rs139876191 with DR in an Italian cohort of type 2 diabetic patients. Our data indicate that the rs139876191 variant was present in 4.8% of patients with PDR compared with 8.3% of diabetic patients without PDR (controls plus NPDR), thus suggesting this variant may confer protection against PDR. Similarly to other studies in this series34, the protective effect of the HMGA1 rs139876191 was confined to PDR only, with no effects on the susceptibility of NPDR. These findings were supported by subsequent studies in vitro, demonstrating the functional significance of the variant allele, which showed a dominant negative effect towards the wild-type allele. As for other similar reports referring to polymorphisms at intron/exon boundaries24, we can hypothesize that the rs139876191 variant could probably affect exonic or intronic splicing regulatory elements, resulting in skipping of exons or other alterations adversely affecting the encoded protein product. Based on the observation that serum VEGFA levels were lower in individuals carrying this variant than in those with the reference allele, it was tempting to hypothesize that the rs139876191 variant, by adversely affecting HMGA1 protein production, could have a role in this reduction. With this in mind, we have performed functional studies with the aim to provide a mechanistic explanation for the protective effect of the rs139876191 variant. Here we report for the first time that HMGA1 is a transcriptional regulator of VEGFA and that HMGA1 is required for the response of VEGFA to hypoxia. Therefore, although other factors in addition to or in concert with VEGFA may contribute to PDR, we can postulate that a deficit of HMGA1 in retinal tissues may protect against hypoxia-induced VEGFA-mediated neovascularization, at least in part through suppression of VEGFA expression. Some of the findings in humans were recapitulated in studies of Hmga1-knockout mice, in which VEGFA was considerably decreased in blood serum and retinal tissue, further supporting the concept that the rs139876191 variant decreases VEGFA expression.

To shed light on the genetic susceptibility to DR, extensive candidate gene studies and linkage analyses have been performed so far, and numerous genetic variants associated with DR have been identified in several genes, including the VEGFA gene8. Although much of these data are controversial, there is evidence that VEGFA plays a key role in the development of retinal neovascularization, and DR in particular. Our data herein well support this view and suggest that abnormalities in VEGFA gene regulation, which may be associated with decreased levels of VEGFA, might occur in patients with PDR, in the absence of mutations within the VEGFA locus.

One strength of the present work is the same ethnic origin of the subjects studied, which minimizes the risk of population stratification. Also, given that diabetic individuals may develop PDR later in life, it is easy to understand how important it can be to define the selection criteria for patients without PDR. In our study, in order to reduce this selection bias, diabetic subjects without PDR were recruited among patients with at least 10 years of duration of diabetes and this, in our opinion, constitutes a further strength of this work. Conversely, as the study design was confined to a single population, the lack of replication studies can be seen as a limitation, and the results unable to be generalized across populations. On the other hand, to our knowledge, there is no available...
American Diabetes Association (ADA) criteria based on fasting glucose levels (second-degree relatives were avoided on the basis of anamnestic data. Diabetes was diagnosed according to European Community Council).

protocols, in accordance with relevant institutional guidelines for animal research (directive n. 86/609/ECC, at the animal facility of the “Istituto Nazionale dei Tumori, Fondazione G. Pascale, Napoli”, using approved animal sent, and the methods were performed in accordance with approved guidelines. All animal work was conducted Sezione Area Centro (protocol registry n. 116 of May 14, 2015). All human participants gave written informed consent, and the methods were performed in accordance with approved guidelines. All animal work was conducted

Study cohort. We studied 1959 consecutive patients with type 2 diabetes, with and without DR, attending the Operative Units of Diabetes (Hospital Pugliese-Ciaccio, Catanzaro) and Endocrinology (University “Magna Gracia”, Catanzaro), between March 2008 and July 2014. All the study subjects were recruited in Calabria (Southern Italy), a region which consists of a population of comparatively limited genetic diversity36. First- and second-degree relatives were avoided on the basis of anamnestic data. Diabetes was diagnosed according to American Diabetes Association (ADA) criteria based on fasting glucose levels (≥126 mg/dL), or glucose levels following a 2 h OGTT (≥200 mg/dL). Patients with type 1 diabetes were excluded on clinical grounds and, in non-unequivocal cases, on the basis of plasma C-peptide levels and negative pancreatic islet-related autoantibodies. In all participants, the diagnosis of DR was based on ophthalmological examination of the ocular fundus after

|                | Carrier n = 37 | Non-carrier n = 97 | P-value |
|----------------|----------------|-------------------|---------|
| Age (yr)       | 54 (47.5–61.5) | 56 (48.5–60.0)    | 0.998   |
| Female         | 18 (48.6)      | 47 (48.5)         | 0.999   |
| BMI (Kg/m²)    | 24 (22.9–25.3) | 24.3 (23.1–25.1)  | 0.901   |
| Systolic BP (mmHg) | 122 (117–130) | 122 (120–130)    | 0.781   |
| Diastolic BP (mmHg) | 75 (70–80)    | 70 (70–80)        | 0.309   |
| FPG (mg/dL)    | 88 (81.5–90)  | 82 (76.5–88)      | 0.774   |
| IL1α (pg/mL)   | 0.0 (0.0–0.3) | 0.0 (0.0–0.3)     | 0.651   |
| IL3 (pg/mL)    | 0.0 (0.0–1.0) | 0.0 (0.0–1.1)     | 0.413   |
| IL2 (pg/mL)    | 3.0 (1.9–3.8) | 3.3 (2.2–5.7)     | 0.079   |
| IL4 (pg/mL)    | 1.5 (1.3–1.8) | 1.3 (1.1–1.6)     | 0.112   |
| IL6 (pg/mL)    | 1.4 (1.2–2.0) | 1.2 (0.9–2.1)     | 0.136   |
| IL8 (pg/mL)    | 2.4 (1.7–4.3) | 2.6 (1.7–4.8)     | 0.356   |
| IL10 (pg/mL)   | 0.5 (0.0–1.1) | 0.7 (0.0–1.3)     | 0.346   |
| VEGF (pg/mL)   | 31.9 (28.1–40.7) | 35.5 (31.6–45.3) | 0.019   |
| IFNγ (pg/mL)   | 0.9 (0.4–2.9) | 1.0 (0.4–2.9)     | 0.753   |
| TNFα (pg/mL)   | 1.6 (1.2–2.2) | 1.8 (1.4–2.4)     | 0.110   |
| MCP1 (pg/mL)   | 178.9 (142.9–232.0) | 212.5 (158.5–243.9) | 0.197   |
| EGF (pg/mL)    | 15.8 (9.5–31.4) | 21.1 (12.9–30.9) | 0.442   |
| VCAM1 (pg/mL)  | 548.9 (439.3–638.4) | 567.8 (462.3–640.5) | 0.519   |
| ICAM1 (pg/mL)  | 240.5 (219.1–273.9) | 248.2 (221.7–279.1) | 0.624   |
| E selectin (pg/mL) | 13.4 (10.1–17.1) | 15.0 (11.2–18.8) | 0.123   |
| P selectin (pg/mL) | 99.0 (90.1–122.6) | 107.2 (92.8–129.0) | 0.126   |
| L selectin (pg/mL) | 938.4 (825.9–1202.0) | 957.3 (844.3–1236.0) | 0.430   |

Table 4. Serum cytokines profile and other serum factors in a healthy population, according to the presence of the rs139876191 variant. Data are medians (IQR) or n (%). Non-parametric Mann–Whitney test was used for distribution comparisons of quantitative variables. The two-tailed Fisher Exact Test was used for proportion comparisons between groups. Significance level < 0.05. BMI, body mass index; FPG, fasting plasma glucose; BP, blood pressure; IL, interleukin; VEGF, vascular endothelial growth factor; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; MCP-1, monocyte chemoattractant protein-1; EGF, epidermal growth factor; VCAM1, vascular cell adhesion molecule 1; ICAM1, intercellular adhesion molecule 1.

genome-wide dataset on DR patients which includes the HMGA1 rs139876191. Among the reasons for this omission, is probably the fact that typically GWAS are designed to exclude variants with a MAF < 5%35. Nevertheless, we want to point out that the present study is clearly not solely an association study between HMGA1 rs139876191 and DR, but of particular interest is the simultaneous demonstration as to how this association translates pathophysio logically, providing a mechanistic explanation for the protective effect of this variant on DR. Therefore, our findings may provide new insights into the molecular mechanisms underlying the development of PDR. Targeting HMGA1 function in VEGFA gene expression might be a novel approach for fighting VEGFA-dependent neovascularization and vascular permeability of retinal vessels. Also, because the HMGA1 rs139876191 defines a specific defect that causes decreased VEGFA, diabetic patients with this variant may have a less severe clinical course of DR than other diabetic patients, and this could not only explain why patients may respond differently to a specific therapy, such as anti-VEGF medicines, but also provide an opportunity for designing tailored treatments based on patient genotype or marker expression. Confirmation of these results in other populations would be important to further validate our findings and their implications in a clinical setting.

Methods

Ethics statements. The study was approved by the local ethics committee, Regione Calabria Comitato Etico Sezione Area Centro (protocol registry n. 116 of May 14, 2015). All human participants gave written informed consent, and the methods were performed in accordance with approved guidelines. All animal work was conducted at the animal facility of the “Istituto Nazionale dei Tumori, Fondazione G. Pascale, Napoli”, using approved animal protocols, in accordance with relevant institutional guidelines for animal research (directive n. 86/609/ECC, European Community Council).

Study cohort. We studied 1959 consecutive patients with type 2 diabetes, with and without DR, attending the Operative Units of Diabetes (Hospital Pugliese-Ciaccio, Catanzaro) and Endocrinology (University “Magna Gracia”, Catanzaro), between March 2008 and July 2014. All the study subjects were recruited in Calabria (Southern Italy), a region which consists of a population of comparatively limited genetic diversity36. First- and second-degree relatives were avoided on the basis of anamnestic data. Diabetes was diagnosed according to American Diabetes Association (ADA) criteria based on fasting glucose levels (≥126 mg/dL), or glucose levels following a 2 h OGTT (≥200 mg/dL). Patients with type 1 diabetes were excluded on clinical grounds and, in non-unequivocal cases, on the basis of plasma C-peptide levels and negative pancreatic islet-related autoantibodies. In all participants, the diagnosis of DR was based on ophthalmological examination of the ocular fundus after
Figure 2. VEGFA gene expression is induced by HMGA1. (a) Human VEGFA-Luc reporter vector (2 μg) was transfected into HepG2 cells, in the presence of increasing amounts (0, 0.5, 1 μg) of HMGA1 effector plasmid, and Luc-activity was measured 48 h later. Data represent means ± s.e.m for three separate experiments; values are expressed as the factors by which Luc-activity increased above the level of the activity obtained in transfections with VEGFA-Luc reporter vector plus the empty effector vector (control), which is assigned an arbitrary value of 1. White bar, mock (no DNA); black bar, pGL3-basic (vector without an insert). *P < 0.05 and **P < 0.01 vs control. (b) qRT-PCR of endogenous VEGFA mRNA from HepG2 (left), and HUVEC (right) cells, pretreated with increasing amounts (100 and 200 pmol) of anti-HMGA1 siRNA or nontargeting control siRNA. (c) VEGFA-Luc-activity and qRT-PCR of endogenous VEGFA mRNA were measured in ARPE-19 cells, under the same conditions as in (a) and (b). WBs of HMGA1 in each condition are shown in the autoradiograms. Lamin A/C and β-Tubulin, controls of protein loading. Cropped blots are shown in the figures. Full-length WBs are presented in Supplementary Fig. S1. *P < 0.05 and **P < 0.001 vs siRNA-untreated (control) cells. (d) Representative VegfA WB of blood serum from wild-type and Hmga1-deficient mice. Densitometric analyses of six to eight independent blots are shown. Black bars, wild-type mice, n = 8; gray bars, Hmga1-knockout mice, n = 6. *P < 0.05 vs wild-type controls. Hmga1 protein expression is shown in fat tissue. All the samples were run under the same experimental conditions. Cropped blots are shown in the figures. Full-length WBs are presented in Supplementary Fig. S1. (e) VegfA mRNA levels in retinal tissue of wild-type (black bars) and Hmga1-deficient (gray bars) mice (n = 6 per genotype), as measured by qRT-PCR. Data are means ± s.e.m of three independent measurements from each animal. *P < 0.05 vs wild-type controls.
dilation of the pupils by experienced ophthalmologists. DR was graded according to the International Clinical Diabetic Retinopathy Disease Severity Scale. Among the patients studied, 436 were classified as having PDR (retinal neovascularization and/or vitreous/preretinal hemorrhage), 587 had NPDR (microaneurysms, retinal hemorrhages, venous beading, retinal edema, hard exudates) and 936 patients were classified as controls with no retinal findings. Patients with pan-retinal photocoagulation were included in the PDR group and the severity of DR was graded based on the worst eye. Ophthalmological examination including fundus examination was carried out by experienced ophthalmologists.

Figure 3. HMGA1 and VEGFA expression in hypoxia. (a) Effect of hypoxia on VEGFA (gray bars) and HMGA1 (black bars) mRNA, in HepG2 cells pre-treated or not with anti-HMGA1 siRNA, as measured by qRT-PCR. Data are means ± s.e.m of three independent experiments, each performed in triplicate. (b) ChIP of the VEGFA promoter gene in HepG2 and ARPE-19 cells, either untreated or pretreated with siRNA against HMGA1, both in normoxic and hypoxic conditions, using an anti-HMGA1 specific antibody (Ab). Representative assays are shown, together with qRT-PCR of ChIP-ed samples. Cropped gels are shown in the figures. Full-length ChIPs are presented in Supplementary Fig. S1.
out annually for all patients. To exclude misclassification due to the short duration of the disease, only patients with at least 10 years duration of diabetes were included in the control group.

**Anthropometric and biochemical measurements.** A series of anthropometric and clinical parameters were evaluated for all patients. Fasting blood samples were collected and biochemical analyses of plasma glucose, triglycerides, total and HDL-cholesterol and serum insulin were performed in all participants with no caloric intake for at least 8 h. LDL-cholesterol was calculated using Friedewald's formula. Serum concentration of 12 different cytokines and growth factors (IL-1α/IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α, MCP-1, VEGFA, EGF), as well as adhesion molecules (E-selectin, P-selectin, L-selectin, VCAM1, ICAM1) was simultaneously determined in a subset of 134 matched healthy subjects29, either carriers or non-carriers of the rs139876191 allele, in the absence of drug treatments, using the biochip analyser Evidence Investigator (Randox Labs).

**Genotyping of the HMGA1 rs139876191 and its functional analysis.** Genomic DNA was extracted from peripheral blood and genotyped for rs139876191 by the fluorescence-based TaqMan allelic discrimination technique (Applied Biosystems)30. Reliability of the TaqMan detection method was confirmed by sequencing analysis of 160 DNA samples from diabetic patients with PDR, 68 DNA samples from patients with NPDR, and 72 DNA samples from control individuals ( Concordance rate >99%), which were directly sequenced for the exon 6 and adjacent introns of the HMGA1 gene (NC_000006.11, http://www.ncbi.nlm.nih.gov). To clarify the functional relevance of the rs139876191 polymorphism (also designated IVS5–13insC), normal and mutant minigenes, spanning exons 5 to 6 of the human HMGA1 gene, which included the C insertion site, were constructed (GenScript) and transiently transfected in HEK-293 cells naturally expressing HMGA1. Cell transfection efficiency was assessed by evaluating the expression levels of the minigenes with real-time PCR using internal controls (GenScript) and transiently transfected in HEK-293 cells.
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