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Association between a *MIR499A* polymorphism and diabetic neuropathy in type 2 diabetes

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ABSTRACT

Aims: Diabetic polyneuropathy (DPN) and cardiovascular autonomic neuropathy (CAN) affect a large percentage of diabetic people and impact severely on quality of life. As it seems that miRNAs and their variations might play a role in these complications, we investigated whether the rs3746444 SNP in the *MIR499A* gene could be associated with susceptibility to DPN and/or CAN.
Methods: We analyzed 150 participants with type 2 diabetes. DNA was extracted from peripheral blood samples and genotyping was performed by TaqMan genotyping assay. Cardiovascular tests, MNSI-Q and MDNS for neuropathic symptoms and signs, VPT, and thermal thresholds were used for CAN and DPN assessment. We performed a genotype-phenotype correlation analysis.
Results: We observed that the GG genotype was associated with a higher risk of developing CAN ($P = 0.002$ and $OR = 16.08$, $P = 0.0005$ and $OR = 35.02$, for early and confirmed CAN, respectively) and DPN ($P = 0.037$ and $OR = 6.56$), after correction for BMI, sex, age, HbA1c and disease duration. Moreover, the GG genotype was associated with worse values of MDNS ($P = 0.017$), VPT ($P = 0.01$), thermal thresholds ($P = 0.01$), and CAN score ($P < 0.001$). A logistic multivariate analysis confirmed that *MIR499A* GG genotype, disease duration and HbA1c contributed to early CAN ($R^2 = 0.26$), while the same variables and age contributed to DPN ($R^2 = 0.21$). With a multiple linear regression, we observed that GG genotype ($P = 0.001$) and disease duration ($P = 0.035$) were the main variables contributing to the CAN score ($R^2 = 0.35$).
Conclusions: We described for the first time that the *MIR499A* genetic variation could be involved in diabetic neuropathies susceptibility. In particular, patients carrying the rs3746444 GG genotype had a higher risk of CAN development, together with a more severe form of CAN.

1. Introduction

Diabetic distal symmetric sensorimotor polyneuropathy (DPN) and diabetic cardiovascular autonomic neuropathy (CAN) are the most common forms of diabetic neuropathies.[1] DPN and CAN affect about 30% and 20% of patients with diabetes, reaching a prevalence of >50% in people with higher age and a longer diabetes duration.[2,3] Moreover, both DPN and CAN impact severely on patients' quality of life, survival and health costs.[2–5] Despite the burden of these complications, their pathogenesis has not been fully explored: it is considered multifactorial, with the interaction between genetic and environmental factors and with hyperglycemia playing a leading role. Furthermore, clinical trials have shown that the development of these complications in any single patient cannot be completely anticipated by the control of hyperglycaemia or of other risk factors.[6] Therefore, the role of genetic factors is crucial.

Indeed, in recent years many studies have highlighted how genetic variations can influence the development of these complications.[7,8] However, very few genes have been extensively investigated in different populations and in large cohorts, among which the *ACE* and *MHTFR* genes.[9–15] In particular, polymorphisms in these two genes have been described as being associated with a higher risk of developing DPN.[13–15] On the contrary, only a few studies have reported genetic associations with CAN: for example, *GSTT1* significantly increased the risk of CAN in a Slovak population,[16] while *TCF7L2* polymorphisms seemed to increase CAN risk in an Italian population.[17]

Recently, we described some associations of microRNA gene polymorphisms with both CAN and DPN susceptibility.[18] MicroRNAs or miRNAs are a class of small RNA molecules that function as regulators of gene expression at post transcriptional level. MiRNA biogenesis starts in the nucleus and concludes in cytoplasm, giving rise from a precursor

Conflicts of interest: none.

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stem loop structure, to two mature miRNAs: the miR-5p in the sense position and miR-3p in the reverse position. A mature miRNA can regulate the expression of several genes through two main mechanisms: the direct degradation of the target mRNA or by interfering with protein translation.[19]

Since their discovery, it has been evident that miRNAs are implicated in the regulation of a plethora of pathways, such as cellular proliferation and differentiation, signal transduction, inflammation and autoimmunity.[20–22] Many studies have investigated miRNA expression profiles in different tissues involved in diabetic pathology, such as the pancreas, adipose tissue, and the liver.[23–25] Several miRNAs have been reported as dysregulated in diabetic patients, such as miR-375, the first miRNA to be identified as a regulator of insulin secretion,[26] miR-1/133a,[27] miR-29,[28] miR-130,[29] and miR-27,[30] all of which are involved in insulin resistance, as well as many other miRNAs.

Although the majority of studies have focused on miRNA expression profiles, new evidence has pointed out that also polymorphisms in their genes could be of interest. In fact, genetic variations in miRNA genes could alter the maturation of miRNAs themselves and the recognition of their targets[31] and therefore, they could also be involved in disease development. Genetic variations in miRNA genes have been found to be associated with several diseases, such as cancer,[32] cardiovascular diseases[33,34] and autoimmune diseases.[35–38] We have recently described the involvement of *MIR128A*, *MIR146A* and *MIR27A* polymorphisms in the risk to develop diabetic neuropathies in an Italian cohort of patients with type 2 diabetes.[18] The variant allele of rs11888095 SNP in *MIR128A* was significantly associated with a higher risk of DPN (OR = 4.89, P = 0.02) and with a higher DPN severity (P = 0.026). The C allele of rs2910164 SNP in *MIR146A* was associated with a lower risk of developing both DPN (OR = 0.49, P = 0.09) and CAN (OR = 0.32, P = 0.052). On the other hand, the variant allele of rs895819 SNP in *MIR27A* was significantly associated with a higher risk of developing early CAN (OR = 3.43 and P = 0.023).

More recently our interest has shifted towards the miR-499, which is specifically expressed in cardiac cells and skeletal muscle.[39–41] MiR-499 has been reported to play a critical role in both cardiac differentiation[39] and cardiac stress response, preventing cardiomyocyte apoptosis by calcineurin-mediated Drp1 activation and consequent mitochondrial fission.[42] The expression of miR-499 was increased at a circulating level after acute myocardial infarction.[43–45] Recently, it has been described as regulating insulin resistance; indeed, an over-expression of miR-499 is able to enhance the glycogen and improve insulin signaling by PTEN inhibition.[46] Moreover, miR-499 expression levels were increased in hearts and nucleus ambiguus of streptozotocin-induced diabetic rats.[47]

Interestingly, miR-499 is transcribed by two genes - *MIR499A* and *MIR499B* - that are located in the same region in the intron of *MYH7B* gene in chromosome 19, but in the opposite direction. Indeed, *MIR499A* is transcribed in the sense direction, while *MIR499B* is transcribed in the opposite direction. Whereas for miR-499a there are expression data, it is not known if miR-499b is expressed.[48] A common single nucleotide polymorphism (SNP), rs3746444 A > G SNP, is located in the corresponding 3p mature miR-499a region. This SNP has been associated with several diseases, such as cardiovascular diseases,[33] autoimmune diseases[49] and cancer.[32]

Thus, we considered the fact that miR-499 is expressed in the heart and brain (nucleus ambiguus), and is involved in both cardiovascular disease and metabolic syndrome/diabetes as a reason to analyse genetic variability of *MIR499A* with respect to diabetic neuropathic complications. Therefore, our aim was to investigate whether the common polymorphism rs3746444 SNP could be associated with susceptibility to DPN and/or CAN. To this end, we analyzed this SNP in the DNA extracted from peripheral blood samples in a population of 150 participants with type 2 diabetes evaluated for CAN and DPN and subsequently performed a genotype–phenotype correlation analysis.

2. Materials and methods

2.1. Patients recruitment

Patients were consecutively recruited from January 2010 to January 2014 among outpatients attending the diabetic clinic of the Policlinico Tor Vergata in Rome (Italy). The inclusion criteria were a diagnosis of type 2 diabetes and age between 18–80 years. The exclusion criteria included presence of peripheral or autonomic neuropathies from causes other than diabetes, conditions potentially responsible for autonomic dysfunction, severe comorbidities (such as malignancies, recent cardiovascular events, heart failure, advanced renal failure or liver disease), advanced peripheral arterial disease, severe psychiatric disorders or any other condition preventing understanding of the questionnaires. From 172 patients initially enrolled, 150 were included according to the selection criteria. The Ethics Committee of the University Hospital of Rome Tor Vergata approved the study. All participants provided written informed consent.

A complete clinical history was recorded regarding diabetes, comorbidity, cardiovascular disease and any potential cause of polyneuropathy. Height, weight, waist circumference, casual blood pressure (BP) and blood glucose at the moment of neurological assessment were measured.

A subject who smoked regularly at least one cigarette per day was considered a current smoker and alcohol consumption was recorded. Subjects who took part in leisure-related physical activity for at least 1 h per week were considered physically active.

Routine laboratory assessment was performed including HbA1c, cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, serum creatinine and 24-h urinary albumin excretion. Micro- and macroalbuminuria were considered present with a 24-h albumin excretion of 30–299 mg or ≥300 mg, respectively. The presence of non-proliferative or proliferative retinopathy was determined via ophthalmoscopic examination. Peripheral arterial disease was considered present on the basis of claudication and/or absence of palpable dorsalis pedis and/or posterior tibial pulses or instrumental reports (Doppler sonography and magnetic resonance angiography) - see Supplementary Tables S1 and S2 for patients' details.

2.2. Assessment of DPN and CAN

Neurological assessment included evaluation of neuropathic symptoms and deficits using the Questionnaire of the Michigan Neuropathy Screening Instrument (MNSI-Q) and the Michigan Diabetic Neuropathy Score (MDNS), respectively.[50] Vibration perception threshold (VPT) was measured using the Biothesiometer (Biomedical instruments, Newbury, OH, USA) at the hallux dorsum and at the lateral malleolus,[51] and age-related normal values derived from literature were used.[52] Cold (CTT) and warm thermal perception thresholds (WTT) were assessed using the Neuro Sensory Analyzer TSA-II (Medoc, Ramat Yishai, Israel) at the dorsum of both feet following the levels test procedure. The definition of DPN (probable) required the presence of at least two abnormalities among neuropathic symptoms, signs, vibration perception threshold, and thermal perception thresholds.[1] The *Douleur neuropathique en 4 questions* (DN4) was also used, as a validated screening tool for neuropathic pain.[53]

We performed four Cardiovascular autonomic reflex tests (CARTs), three based on heart rate response to deep breathing, lying to standing, and to Valsalva manoeuvre, and the orthostatic hypotension test, according to standard procedure and using age-related reference values.[53] An autonomic score was calculated by giving a score of 0 for a normal result, 1 for a borderline result and 2 for an abnormal result (range 0–8). We considered patients with ≥1 abnormal cardiovagal test as having early CAN and those with ≥2 abnormal tests as having confirmed CAN.[1,53]

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