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Influence of genetic polymorphisms in *DICER* and *XPO5* genes on the risk of coronary artery disease and circulating levels of vascular miRNAs

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ABSTRACT

Introduction: Single-nucleotide polymorphisms (SNPs) in microRNA (miRNA) machinery genes may affect the regulatory capacity of miRNAs by impacting their biogenesis. The aim of the study was to analyze the association between SNPs in two key genes (*DICER rs1057035T > C* and *XPO5 rs11077A > C*) and coronary artery disease (CAD) risk as well as to examine their effects on circulating levels of vascular miRNAs.

Materials and methods: Within the Italian GENOCOR cohort, we studied a cohort of 557 patients (502 males, 57 ± 9 years) with angiographically documented CAD. A total of 443 healthy controls (262 males, 56 ± 12 years) was also enrolled. Genotyping was determined by using a TaqMan®SNP genotyping assay. Analysis of miR-132 and miR-140-3p was assessed in a subset of 70 CAD patients by using qRT-PCR.

Results: There were statistically significant differences between CAD patients and healthy controls in the distribution of both *DICER* and *XPO5* genotypes (p = 0.03 and p = 0.02, respectively). Multivariate analysis showed a significantly decreased risk of CAD by 50% in patients with *DICER rs105703CC* genotype as compared to *TC* heterozygote and *TT* homozygote patients ($OR_{adjusted}$ = 0.50; CI: 0.30–0.83, p = 0.007). In a recessive model, the *XPO5 rs11077*CC genotype was associated with a 32% reduced risk of CAD ($OR_{adjusted}$ = 0.68; CI: 0.30–0.99 p = 0.047). *XPO5 rs11077*CC genotype was significantly associated with higher levels of both miRNA-132 (p = 0.04) and miRNA-140-3p (p = 0.03).

Conclusions: Genetic polymorphisms in *DICER* and *XPO5* genes are associated with a decreased risk of CAD, probably by impacting expression levels of vascular and cardiac-specific miRNAs. Further studies are needed to better elucidate the biological relevance of both variants in CAD development.

1. Introduction

Better prevention and management of coronary artery disease (CAD) and its complications represent a major challenge for health care systems. Identifying novel risk markers may improve risk stratification and the development of novel treatment strategy.

Recently, there has been much interest in the role of micro-RNAs (miRNAs) in cardiovascular biology [1]. miRNAs are a class of small noncoding RNAs that regulate several processes in cardiovascular disease (e.g., myocardial remodeling and fibrosis, vascular inflammation, lipid processing, and electric remodeling) by mediating posttranscriptional gene silencing [2].

Accordingly, recent studies have shown the potential utility of several circulating miRNAs as cardiac biomarkers [3]. However, there are still several major limitations in using circulating miRNAs in clinical routine, due to contrasting results among studies, technical and analytical difficulties and unexplored effects of influencing confounding factors [4].

Currently, there are also fundamental gaps in our knowledge of the impact of genetic variants on miRNA biogenesis and function. Single nucleotide polymorphisms (SNPs) can have profound effects on miRNA functionality at all levels, including miRNA transcription, maturation and target specificity [5], and as such they can also affect the occurrence and prognosis of CAD [6].

Recently, clinical studies have shown that functional SNPs in miRNA genes might be associated with increased risk of CAD development [7]. However, SNPs within the seed regions of evolutionarily conserved miRNAs are rare and unlikely to be important for function [8].

Conversely, SNPs within the biogenesis pathway of miRNAs (or

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miRNA machinery genes) may affect the regulatory capacity of miRNAs by affecting their biogenesis and processing [6].

Although largely lacking in functional validation, several recent studies have supported that these SNPs are associated with development and progression of several types of cancers, such as lung cancer, esophageal cancer, breast cancer, bladder cancer and renal cell carcinoma [9].

Therefore, it is plausible that SNPs in the miRNA biogenesis pathway genes may influence their activity, playing an important role in CAD risk as well. However, to date, no studies have assessed these associations in patients with a CAD [6].

To test this hypothesis, the purpose of this study was to analyze the association between SNPs in two miRNA processing genes, *DICER rs1057035 T > C* and *XPO5 rs11077 A > C*, and CAD risk as well as to examine their effects on circulating levels of two vascular miRNAs (miR-132 and miR-140-3p), whose prognostic and risk predictive potential was recently shown in patients with cardiovascular disease [10–12].

2. Materials and methods

2.1. Study population

In the Italian GENOCOR (Genetic Mapping for Assessment of Cardiovascular Risk) study [13], we selected a group of 557 patients (502 males, 57 ± 9 years) who were admitted in our Institute with known or suspected stable CAD. Inclusion criteria were documentation of CAD defined as angiographically significant coronary stenosis in at least one major vessel diseased (> 50% lumen reduction). The severity of CAD was determined as the number of affected vessels (one-, two-, or three-vessel disease).

A group of 443 ethnically-matched and healthy controls (262 males, 56 ± 12 years) members of the medical and technical staff of our institution, and negative for any clinical evidence of cardiovascular disease, was used as control group. For all patients and controls data on smoking status, arterial hypertension (systolic blood pressure > 140 mmHg and/or diastolic pressure > 90 mmHg), hypercholesterolemia (plasma cholesterol > 220 mg/dl), diabetes (fasting plasma glucose > 125 mg/dl or glucose-lowering drugs), and BMI were collected and coded in a dichotomized fashion.

Written informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Comitato Etico Sperimentazione Farmaco – Azienda Ospedaliera Universitaria Pisana, Italy). ClinicalTrials.gov Identifier is NCT01506999 (January 10, 2012).

2.2. Genotyping assays

Blood samples were collected from all participants and genomic DNA was extracted by using the QIAGEN BioRobot® EZ1 System. DNA concentration and quality were assessed by a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, USA), and an absorbance ratio at both 260 and 280 nm (A260/A280) > 1.6 was considered suitable for the subsequent analysis.

Allelic discrimination for the *DICER rs1057035 T > C* and *XPO5 rs11077 A > C* polymorphisms was completed by quantitative real-time PCR (qRT-PCR) on CFX RT-PCR System (Bio-Rad) by using the TaqMan® SNP Genotyping assays (Applied Biosystems, USA). Negative and positive controls were included as a quality control measure. Genotyping results were analyzed by allelic discrimination assay of CFX Manager® software (Bio-Rad).

2.3. Analysis of miR-132 and miR-140-3p expression by qRT-PCR

miRNA expression levels were analyzed in a random subset of 70 patients with CAD by using qRT-PCR assay, as previously described [14]. Total RNA was extracted from plasma samples using the miRNeasy Serum/Plasma Kit (Qiagen, Milan, Italy) according to the manufacturer's recommendations. The concentration and purity of total RNA samples was evaluated by measuring the absorbance at 260 and 230 nm in a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Extracted total RNA was reverse transcribed into cDNA by using TaqMan miRNA Reverse Transcription Kit using TaqMan Universal PCR Master Mix, no AmpErase UNG (Thermo Fisher Scientific). qRT-PCR quantification of miR-132 and miR-140-3p was performed with a TaqMan MicroRNA Assay (Thermo Fisher Scientific, Waltham, MA, USA). Each reaction was run in triplicate in 384-well plate CFX RT-PCR system and the output data were analyzed by using CFX Manager software (Bio-Rad, Milan, Italy). The expression levels of each miRNA were normalized to U6 snRNA by using the ΔΔCt method.

2.4. Statistical analysis

Statistical analyses of the data were conducted with the StatView statistical package, version 5.0.1 (Abacus Concepts, Berkeley, CA, USA). Values are presented as mean ± standard deviation (SD) or percent. Comparisons of normally distributed variables between groups were performed by using unpaired *t*-tests. For the comparison of circulating miRNA levels, statistical analysis was performed using log-transformed normalized CT value. Chi-square test was used to test for deviation from Hardy–Weinberg equilibrium and to compare allelic and genotypic frequencies between groups. The most significant test among the different genetic models (dominant, additive or recessive) was used to determine the statistical significance of each SNP. Univariate and multivariate logistic regression analysis was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association of the *XPO5* and *DICER* polymorphisms and CAD risk. The false-positive discovery rate (FDR) correction was used to adjust multiple comparison tests. Based on our sample size, the study had an 80% power to detect an effect of OR ≥ 1.5 for a risk allele frequency ≥ 0.3. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Clinical and genetic characteristics of study subjects

The characteristics of the study participants were summarized in Table 1. There were no statistically significant differences between cases and controls in terms of age. Significant differences were found for gender and traditional risk factors.

Minor allele frequency (MAF) of *DICER rs1057035 T > C* and *XPO5 rs11077 A > C* in controls was compatible with MAF reported in public available resources (dbSNP; in 1000 Genomes Project) and various European populations. The distribution of observed genotype frequency of the two SNPs in both control subjects and CAD patients was also compatible with the Hardy–Weinberg equilibrium (p values > 0.05; Table 1), providing no evidence of population stratification within the dataset. Table 2 summarized the association of *DICER rs1057035 T > C* and *XPO5 rs11077 A > C* SNPs and CAD risk under different genetic models. After adjustment for age, gender and traditional risk factors, the risk of CAD was decreased by 50% in the patients with *DICER rs105703 CC* genotype as compared to *TC* heterozygote and *TT* homozygote patients ($OR_{adjusted}$ = 0.50; CI: 0.30–0.83, p = 0.007). The association remained statistically significant after controlling for multiple comparisons using the FDR correction. In a recessive model, the *XPO5 rs11077CC* genotype was also associated with a 32% reduced risk of CAD ($OR_{adjusted}$ = 0.68; CI: 0.30–0.99 p = 0.047).

Table 1
Clinical and genetic characteristics between control subjects and CAD patients.

	Healthy controls (n = 443)	CAD patients (n = 577)	P value
Mean ± SD age, yrs	56 ± 12	57 ± 9	0.2
Gender, male n (%)	262(59)	502 (87)	< 0.0001
Smoking habit, n (%)	199(45)	354(61)	< 0.0001
Hypertension, n (%)	103 (23)	249 (43)	< 0.0001
Dyslipidaemia, n (%)	103(23)	394 (68)	< 0.0001
Diabetes mellitus, n (%)	17 (4)	81(14)	< 0.0001
BMI, kg/m^2, mean ± SD	24 ± 2	27 ± 5	< 0.0001
DICER rs1057035 T > C genotype, n (%)			
TT	207 (47)	276 (48)	0.03
TC	180 (40)	256 (44)	
CC	56 (13)	45 (8)	
HWE-p	0.1	0.2	
XPO5 rs11077 A > C genotype, n (%)			
AA	145 (33)	205 (35)	0.02
AC	203 (46)	287 (50)	
CC	95 (21)	85 (15)	
HWE-p	0.1	0.3	

HWE: Hardy-Weinberg equilibrium.

Table 2
Association of *DICER* and *XPO5* polymorphisms and CAD risk under different genetic models.

SNP	Genotype	OR* (95% CI)	P value	FDR-p value
DICER rs1057035 T > C				
Codominant	*TT*	1		
	TC	0.94 (0.64–1.29)	0.7	0.7
	CC	0.49 (0.29–0.83)	0.008	0.009
Dominant	*TT*	1		
	TC + CC	0.83(0.61–1.12)	0.2	0.3
Recessive	*TT + TC*	1		
	CC	0.50 (0.30–0.83)	0.007	0.008
XPO5 rs11077 A > C				
Codominant	*AA*	1		
	AC	1.03 (0.73–1.43)	0.88	0.88
	CC	0.70 (0.46–1.09)	0.11	0.12
Dominant	*AA*	1		
	AC + CC	0.92 (0.67–1.27)	0.62	0.68
Recessive	*AA + AC*	1		
	CC	0.68 (0.30–0.99)	0.047	0.05

Adjusted for age, gender and traditional vascular risk factors. False positive discovery rate (FDR)-adjusted p-value.

3.2. Subgroup analyses of DICER rs1057035 T > C and XPO5 rs11077 A > C polymorphisms on CAD risk

We further analyzed the associations of *DICER rs1057035 T > C* and *XPO5 rs11077 A > C* with CAD risk by stratified analyses according to gender, smoking status, hypertension, dyslipidemia, diabetes mellitus (Table 3). In these analyses, it was demonstrated that the *DICER rs1057035 T > C* polymorphism showed a significant protective effect in the males (p = 0.006), smokers (p = 0.003), hypertensive (p = 0.03) and dyslipidemic patients (p = 0.005). There was no evidence of significant association between *XPO5 rs11077 A > C* polymorphisms and CAD risk among all subgroups.

3.3. Association between DICER rs1057035 and XPO5 rs11077 polymorphisms and the circulating expression of miRNA-132 and miRNA-140-3p

In order to investigate the functional relevance of the *DICER rs1057035* and *XPO5 rs11077* polymorphisms, we analyzed the association between the genotypes and circulating levels of two vascular miRNAs (miRNA-132 and miRNA-140-3p) in a subset of CAD patients. There were no significant correlations between the plasma levels of miRNA-132 and miRNA-140-3pand any of the traditional risk factors as well as severity of CAD (data not shown). Plasma expression levels of miRNA-132 and miRNA-140-3p were lower in carriers with *DICER rs105703 CC* genotype as compared to *TC* heterozygote and *TT* homozygote patients, but without statistical significance (Fig. 1a). On the contrary, the *XPO5 rs11077 CC* genotype was found to be significantly associated with higher levels of both miRNA-132 (p = 0.04) and miRNA-140-3p (p = 0.03) as compared to carriers with wild-type *AA* and *AC* genotypes (Fig. 1b).

Table 3
Stratified effects of *DICER rs1057035 T > C* and *XPO5 rs11077 A > C* on CAD risk.

	DICER rs1057035 T > C Controls/cases, n		OR[a] (95% CI) p value	*XPO5 r11077 A > C* Controls/cases, n		OR[a] (95% CI) p value
	TT + TC	*CC*		*AC + AC*	*CC*	
Gender						
Male	226/464	36/38	0.46 (0.26–0.79) p = 0.006	201/425	61/77	0.73 (0.48–1.12) p = 0.16
Female	161/68	20/7	0.72 (0.24–2.11) p = 0.72	147/67	34/8	0.52 (0.20–1.33) p = 0.17
Smoking habit						
No	218/204	26/19	0.73 (0.35–1.54) p = 0.41	185/191	59/32	0.57 (0.32–1.02) p = 0.059
Yes	169/328	30/26	0.35 (0.18–0.69) p = 0.003	163/301	36/53	0.81 (0.47–1.39) p = 0.44
Hypertension						
No	299/299	41/29	0.59 (0.32–1.08) p = 0.09	265/273	75/55	0.70 (0.44–1.13) p = 0.15
Yes	88/233	15/16	0.39 (0.16–0.92) p = 0.03	83/219	20/30	0.64 (0.31–1.32) p = 0.23
Dyslipidemia						
No	304/167	36/16	0.81 (0.41–1.59) p = 0.54	266/148	74/35	0.82 (0.50–1.35) p = 0.44
Yes	83/365	20/29	0.36 (0.18–0.73) p = 0.005	82/344	21/50	0.57 (0.3–1.06) p = 0.07
Diabetes mellitus						
No	375/455	51/41	0.59 (0.35–0.99) p = 0.04	337/422	89/74	0.72 (0.48–1.07) p = 0.11
Yes	12/77	5/4	0.12 (0.02–0.58) p = 0.008	11/70	6/11	0.37 (0.10–1.38) p = 0.14

[a] Adjusted for confounders.

4. Discussion

To the best of our knowledge, this is the first case-control study to investigate the association between *DICER rs1057035 T > C* and *XPO5 rs11077 A > C* polymorphisms and risk of CAD. Our results showed that both SNPs was significantly associated with a reduced CAD risk. Interestingly, a more pronounced protective effect of *DICER rs1057035 T > C* polymorphism was observed in the presence of several traditional risk factors, supporting indirectly the notion that miRNAs may be involved in the vascular modulation of atherogenic risk factors [15]. Further, our findings showed that *XPO5 rs11077CC* genotype was

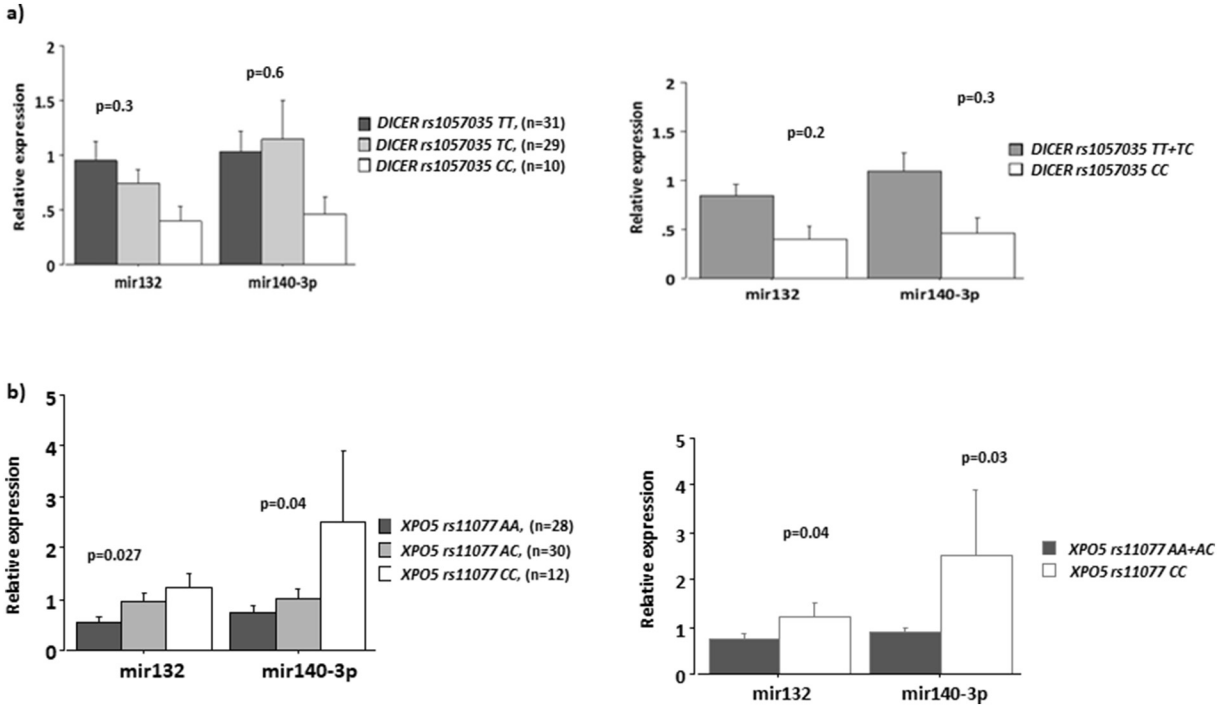

Fig. 1. Influence of *DICER rs1057035* and *XPO5 rs11077* polymorphisms on circulating expression levels of miR-132 and miR-140-3p in a subset of CAD patients.

significantly correlated to higher levels of two mature cardiovascular miRNAs, miRNA-132 and miRNA-140-3p, known to be deregulated in CAD patients [10–12].

DICER is an enzyme responsible for the cleavage of all pre-miRNAs into their mature form, and it has previously been implicated in the cell dysregulation of several cancers [16–18].

Recently, experimental evidence has also investigated the role of Dicer in atherosclerosis [19,20]. Notably, reduced endothelial expression of the DICER decreased monocyte adhesion, endothelial chemokine ligand 1 expression, atherosclerosis and the lesional macrophage content in apolipoprotein E knockout mice (Apoe−/−)after exposure to a high-fat diet in part by reducing miRNA-103 expression [19]. In contrast to its role in endothelium, Dicer deletion in macrophages induced advanced atherosclerosis by increasing mitochondrial fatty acid degradation in foam cells, demonstrating a differential role of DICER depending on different cell types [20].

The *rs1057035 T > C* polymorphism is located within 3′-untranslated region (UTR) of *DICER* gene and it has reported that *rs1057035* variant *C* allele could lead to significantly lower expression levels of enzyme as compared to the *T* allele [21]. Accordingly, several case-control studies have examined the association between *DICER rs1057035* polymorphism and cancer risk, and two meta-analyses found that *C* allele significantly decreases cancer risk [22,23].

XPO5 is a protein responsible for the transport of pre-miRNAs between the nuclear and cytoplasmic compartments, and knocking down its expression leads to reduced miRNA levels [24].

The *rs11077* SNP in the *XPO5* gene has been associated with the risk of developing esophageal cancer, colorectal cancer and renal carcinoma, and it also appears to be related to better outcomes in multiple myeloma and non-small cell lung cancer patients [9,25,26].

Our results revealed that both minor alleles of *DICER* and *XPO5* have a protective role against CAD, likely by impacting expression levels of mature miRNAs and, consequently, their function of gene regulation.

However, due to the complexity of miRNA biogenesis, the functional biological consequences of these SNPs remain difficult to determine.

SNPs in 3′UTR of genes may interfere with mRNA stability by affecting regulatory protein-mRNA and miRNA-mRNA interactions. Accordingly, the C allele of *DICER rs1057035* polymorphism seems to elevate binding of has-miR-574-3p, which has been identified as a candidate tumor promoter miRNA, leading to decrease the expression of the DICER gene and, thus, contributes to decrease the risk of cancer [27].

Moreover, a previous study showed that*XPO5 rs11077 CC* genotype is associated with reduced expression in a Renilla luciferase 3′UTR reporter system [26].

Consistent with a previous research [28], our findings now showed that *XPO5 rs11077 A > C* SNP *rs11077* was positively associated with significant increased expression levels of circulating mature miRNAs.

However, some limitations of our study merit consideration. Firstly, this study was a hospital-based case-control study and is limited by the lack of an independent validation set of patients. Secondly, the study did not evaluate the relationship between other potentially functional SNPs in miRNA processing genes and CAD risk. Finally, we performed qRT-PCR expression of only two mature circulating miRNAs in a subset of patients without healthy controls, and it must be considered as explorative and hypotheses generating. Nevertheless, we focused our interest on patients with CAD to find any significant differences in circulating cardiovascular miRNA in relation to genotypes.

In conclusion, this is the first study showing that genetic variations in the 3′-UTR of *DICER* and *XPO5* genes are associated with a reduced risk of CAD, probably by impacting expression levels of circulating vascular and cardiac-specific miRNAs.

Experimental studies, including analysis on cell lines or tissues, are needed to better define the biological relevance of *DICER rs1057035 T > C* and *XPO5 rs11077 A > C* polymorphisms. Further studies are also necessary to validate our findings in larger populations and another ethnic groups.

Declaration of Competing Interest

None.

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