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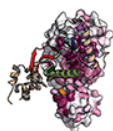
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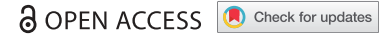View related articles

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Single nucleotide polymorphism rs13079080 is associated with differential regulation of the succinate receptor 1 (SUCNR1) gene by miRNA-4470

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ABSTRACT

Oxidative stress is a feature of many common diseases. It leads to excessive formation and subsequent release of the mitochondrial metabolite succinate, which acts as a signalling molecule through binding the succinate receptor (SUCNR1). Recently, a potential role for SUCNR1 was proposed in age-related macular degeneration (AMD), a common cause of vision loss in the elderly associated with increased oxidative stress. Here, we evaluated the potential effect of genetic variants in *SUCNR1* on its expression through differential micro-RNA (miRNA) binding to target mRNA, and investigated the relevance of altered *SUCNR1* expression in AMD pathogenesis.

We analysed common *SUCNR1* SNPs for potential miRNA binding sites and identified rs13079080, located in the 3′-UTR and binding site for miRNA-4470. Both miRNA-4470 and *SUCNR1* were found to be expressed in human retina. Moreover, using a luciferase reporter assay, a 60% decrease in activity was observed when miRNA-4470 was co-expressed with the C allele compared to the T allele of rs13079080. Finally, genotyping rs13079080 in an AMD case-control cohort revealed a protective effect of the TT genotype on AMD compared to the CC genotype (p = 0.007, odds ratio = 0.66). However, the association was not confirmed in the case-control study of the International AMD Genomics Consortium.

Our study demonstrates that the T allele of rs13079080 in *SUCNR1* disrupts a binding site for miRNA-4470, potentially increasing *SUCNR1* expression and consequently increasing the capacity of sensing and dealing with oxidative stress. Therefore, it would be worthwhile assessing the relevance of rs13079080 in other oxidative stress-associated diseases in future studies.

Introduction

Oxidative stress is a shared feature of many common diseases, such as diabetes mellitus (DM), chronic kidney disease and cancer, and is known to coincide with alterations in the tricarboxylic acid (TCA) cycle, leading to excessive formation and subsequent release of the mitochondrial metabolite succinate [1]. Recently, it became apparent that released succinate can act as a signalling molecule through binding to its GPR91 plasma membrane receptor, subsequently renamed the succinate receptor (SUCNR1). This receptor couples disturbances in energy metabolism to adaptive responses such as blood pressure regulation and inducing the release of extracellular factors [2,3].

The *SUCNR1* gene is expressed in several tissues including the retinal pigment epithelium (RPE) [4], the proximal and distal tubules of the kidney [2,5] and dendritic cells [2,6]. *Sucnr1* knockout mouse studies revealed that Sucnr1 activation in the kidney confers hypertension in induced DM type-1 [5,7]. Furthermore, we recently observed that *Sucnr1* knockout mice are less diabetic compared to their wild-type littermates after exposure to a high-fat diet [8]. Consequently, SUCNR1 modulators are considered as highly promising

novel pharmaceuticals to treat these common diseases [2]. However, the role of SUCNR1 in these disorders is poorly understood and a clear role in human (patho)physiology has never been demonstrated. Recently, however, a potential role for SUCNR1 was proposed in age-related macular degeneration (AMD) [9].

AMD is the leading cause of vision impairment in elderly people in the Western world, and its prevalence is expected to rise to 200 million individuals by the year 2020 due to the exponential aging of the population [10]. AMD is a degenerative disease of the macula and is characterized by extracellular deposits known as drusen. Drusen contain lipids, lipoproteins and inflammatory factors [11,12], are found between the RPE layer and the Bruchs' membrane [13], and are associated with reduced functionality of the RPE layer [14]. Advanced AMD is classified in two forms based on their pathological features. The first form is 'dry' AMD, and is characterized by RPE and photoreceptor cell loss known as geographic atrophy [15]. Due to extensive neovascularization of the choroidal microvascular bed, the second form is called 'wet' AMD [15]. A known stimulator for choroidal neovascularization is vascular endothelial growth factor (VEGF) [16]. Wet AMD patients can be treated by targeting VEGF by

intraocular injections of anti-VEGF, which has led to a significant improvement in visual acuity [17].

Increased oxidative stress is considered to be a key factor underlying AMD pathogenesis. The macula is susceptible to oxidative stress due to its high metabolic activity, high oxygen pressure from the choroidal blood flow, and exposure to bright light [18]. Moreover, in mice, the Sucnr1 has been linked to AMD, as stress-induced activation of Sucnr1 has been shown to increase Vegfa production in the retina [19]. Furthermore, Sucnr1 knockout mice developed lipid accumulations between the RPE and Bruchs' membrane, resembling drusen seen in AMD [9]. After reintroduction of the Sucnr1 into the immune system of the knockout mice, infiltration of macrophages into the retina and neovascularization was observed [9]. Additionally, an association of a single nucleotide polymorphism (SNP) in an intron of the SUCNR1 gene with AMD was reported [9], further supporting a potential role for SUCNR1 in AMD pathogenesis.

Since 25 years, it has been realized that expression of genes can be regulated by small RNA molecules later termed micro-RNAs (miRNA) [20] and it is suggested that over a third of the genes in the human genome are regulated by miRNAs [21]. MiRNAs regulate gene expression post-transcriptionally [22] by binding the 3′-UTR region of mRNAs after which the mRNA is targeted for RISC-mediated degradation or translational arrest occurs [23]. In this study we aimed to evaluate the effect of genetic variants on regulation of SUCNR1 through miRNA binding alteration, and to investigate the relevance of altered SUCNR1 expression in AMD pathogenesis. We indeed identified a SNP in the 3′-UTR of the SUCNR1 gene and this transcript predicted to disrupt a binding site for miRNA-4470, which was predicted to be expressed in the retina by previous studies [24]. The expression of the miRNA and SUCNR1 in the retina were assessed and the effect of miRNA-4470 on SUCNR1 mRNA expression was further investigated. Additionally, the association of the SNP with AMD was analysed in an AMD case-control cohort.

Results

SNPs in the SUCNR1 gene affecting miRNA binding sites

To determine whether SUCNR1 expression could be influenced by genetic variants leading to altered miRNA binding, we analysed all SNPs (minor allele frequency (MAF) >0.01) in the SUCNR1 gene for potential miRNA binding sites. In silico analysis of the SUCNR1 gene revealed 48 common SNPs, of which 35 SNPs (73%) were found in intronic regions and 12 SNPs (25%) were found in the 3′-UTR (Table 1). Only one SNP was found in the coding region but was located at the 5′-end of exon 3, a location that has never been reported to be regulated by miRNAs. Analysis of the SNPs found in the 3′-UTR with the online available mirSNP tool [25] revealed four SNPs that are predicted to affect binding of miRNAs (Table 2). In total, 12 miRNAs were found of which the binding was predicted to be affected by these four SNPs. The effect of the minor allele compared to the major allele of each SNP ranged from creating a miRNA binding site (create), disrupting a miRNA binding

site (break), to creating a stronger or less strong miRNA binding (enhance and decrease, respectively).

Expression of miRNA-4470 and SUCNR1 in the retina

To assess the potential relevance of the four miRNA binding sites in AMD pathogenesis, the expression of the miRNAs and SUCNR1 in the human retina and RPE was determined. Evaluation of online-available RNA-seq databases revealed that, in line with literature [4], SUCNR1 is expressed in human retina and significantly more in RPE-choroid-sclera tissue ($p = 6.63 \times 10^{-5}$, Figure 1A). Of the potential SUCNR1-regulating miRNAs, only miRNA-4470 was found to be significantly higher expressed in RPE cells derived from human embryonic or induced pluripotent stem cells ($p = 0.03$, Figure 1B and figure S1, respectively) compared to non-differentiated stem cells. Because miRNA-4470 was only reported to be expressed in in vitro derived RPE cells, a condition known to often change RNA expression levels as compared to in vivo, we tested two primary human retina-RPE-choroid samples for miRNA-4470 expression. Monocyte derived dendritic cells (moDCs) were used as a positive control because these cells have been shown to express miRNA-4470 [26]. Indeed, the expected 59-basepair product was amplified from RNA derived from primary retina-RPE-choroid samples as well as the moDC positive control, but not from their reverse transcriptase (-RT) negative control (Figure 1C). Subsequent sequence analysis of these fragments confirmed that the product corresponded to miRNA-4470 (not shown). These data revealed that the SUCNR1 gene and miRNA-4470 are both expressed in the human retina.

Regulation of SUCNR1 expression by miRNA-4470 depending on the rs13079080 allele

In silico evaluation of SNP rs13079080 revealed that the major C allele is predicted to form a miRNA-4470 binding site in the 3′-UTR of the SUCNR1 gene transcript (Figure 2A). When the minor T allele is present in the 3′-UTR of the SUCNR1 gene transcript, the micro-RNA binding site is predicted to be disrupted (Figure 2A). To assess whether miRNA-4470 indeed decreases the mRNA levels of the SUCNR1 gene transcript carrying the major C allele, but not in the presence of the minor T allele of rs13079080, a luciferase assay was carried out. The 3′-UTRs were cloned in luciferase assay reporter constructs and tested for binding and degradation by miRNA-4470 or a scrambled negative control miRNA following transfection in HEK293 cells. Indeed, miRNA-4470 co-transfection resulted in a significant decrease in luciferase activity derived from the C allele construct as compared to the scrambled miRNA co-transfection condition ($p = 2.25 \times 10^{-5}$), whereas no effect was observed when miRNA-4470 was co-transfected with the T allele construct or the empty vector (Figure 2B). A 60% reduction in luciferase activity was observed when comparing both alleles co-transfected with miRNA-4470. Co-transfection with the scrambled miRNA did not affect luciferase activity derived from of either construct. These data indicate that the expression of the SUCNR1 with the C, but not the T, allele is indeed affected by miRNA-4470.

Table 1. SNPs in *SUCNR1* gene.

SNP	Change in allele	Allele frequency	Location in the gene	Chromosome location
rs1445359	A/G	0.960863/0.039137	intron	151873953
rs76861810	C/T	0.981230/0.018770	intron	151874048
rs13090483	C/T	0.023363/0.976637	intron	151874117
rs9855643	A/G	0.489816/0.510184	intron	151874810
rs76992305	A/G	0.968850/0.031150	intron	151875177
rs78598704	C/T	0.955072/0.044928	intron	151875205
rs1445358	A/G	0.151158/0.848842	intron	151875430
rs79020368	C/G	0.044928/0.955072	intron	151875605
rs77221620	G/T	0.968850/0.031150	intron	151875836
rs78279353	A/T	0.014377/0.985623	intron	151875973
rs9828478	G/T	0.739417/0.260583	intron	151876506
rs1520211	C/G	0.868610/0.131390	intron	151876532
rs1520212	A/T	0.868610/0.131390	intron	151876533
rs1520213	G/T	0.131589/0.868411	intron	151876768
rs112099950	C/T	0.027756/0.972244	intron	151876775
rs1520214	A/G	0.868411/0.131589	intron	151876831
rs9824780	C/T	0.261781/0.738219	intron	151877081
rs1476177	A/T	0.486022/0.513978	intron	151877667
rs80251978	C/T	0.043331/0.956669	intron	151877817
rs78717519	C/T	0.010982/0.989018	intron	151878205
rs75824320	A/G	0.031150/0.968850	intron	151878550
rs62272956	A/C	0.989018/0.010982	intron	151878552
rs7638353	A/C	0.512181/0.487819	intron	151878612
rs12632021	G/T	0.132388/0.867612	intron	151878616
rs9811297	A/C	0.098043/0.901957	intron	151878800
rs79257023	C/T	0.031150/0.968850	intron	151878919
rs2030370	C/T	0.525160/0.474840	intron	151878997
rs12486484	C/T	0.486621/0.513379	intron	151879385
rs1850963	A/T	0.486821/0.513179	intron	151879460
rs13315275	A/G	0.340256/0.659744	intron	151879522
rs58487215	C/T	0.050719/0.949281	intron	151879695
rs73168928	C/T	0.116014/0.883986	intron	151880081
rs75265510	A/G	0.946086/0.053914	intron	151880183
rs34882247	C/T	0.981430/0.018570	intron	151880354
rs16863710	C/T	0.971518/0.028474	coding-synon	151880681
rs200415012	A/C	0.014376/0.985624	intron/nonsense	151880687
rs13079080	C/T	0.584065/0.415935	intron/untranslated-3	151881605
rs56171835	C/T	0.859225/0.140775	intron/untranslated-3	151881694
rs73168929	A/C	0.065096/0.934904	intron/untranslated-3	151881896
rs73018440	G/T	0.071486/0.928514	intron/untranslated-3	151881934
rs73168930	C/T	0.140575/0.859425	intron/untranslated-3	151882256
rs62274881	A/C	0.013379/0.986621	intron/untranslated-3	151882419
rs71306564	A/C	0.904952/0.095048	intron/untranslated-3	151882614
rs60324356	C/T	0.928714/0.071286	intron/untranslated-3	151882995
rs6763399	C/T	0.014177/0.985823	intron/untranslated-3	151883648
rs34505768	A/T	0.973043/0.026957	intron/untranslated-3	151883894
rs73168931	A/C	0.974042/0.025959	intron/untranslated-3	151884002
rs75790719	A/C	0.946885/0.053115	intron/untranslated-3	151884195

SNPs in *SUCNR1* extracted from UCSC human genome browser using the Human Dec. 2013 (GRCh38/hg38) assembly and the dbSNP151 track.

Association analysis of rs13079080 with AMD

To assess whether the SNP (rs13079080) in the miRNA-4470 binding site is associated with AMD development, we genotyped this SNP in a cohort of 1,784 individuals, of which 848 were graded as AMD (including all stages) and 936 as controls. Demographics of the EUGENDA case-control cohort is described in Table 3. In this case-control cohort, the SNP rs13079080 was associated with AMD (p = 0.008, adjusted for age and sex). The association was driven by the homozygous alternative genotype TT, which was associated with a reduced risk for AMD when compared to the homozygous reference CC genotype (p = 0.007, OR = 0.66, 95% CI = 0.49–0.89). The heterozygous genotype CT was not associated with AMD (p = 0.780, OR = 1.03, 95% CI = 0.83–1.26).

We additionally explored whether this association was identified in the largest GWAS on advanced AMD published to date [27]. This study included 16,144 advanced AMD patients and 17,832 controls from 25 different cohorts and analysed >12 million genetic variants. Here, SNP rs13079080

was not associated with advanced AMD in the pooled analysis (p = 0.54, OR = 1.01, 95% CI = 0.98–1.04). A more detailed analysis of this SNP in 22 of the assessed cohorts showed a nominal association (p < 0.05) for the Edinburgh cohort and no association for the remaining cohorts. However, the direction of the effect in the Edinburgh cohort was the opposite compared to the effect observed in the EUGENDA case-control cohort (p = 0.03, OR = 1.36, 95%CI = 1.03–1.79, Table S1). These data indicate that rs13079080 was associated with AMD in the EUGENDA cohort, but this association was not confirmed in the IAMDGC dataset.

Discussion

In this study, we identified a SNP (rs13079080) in the 3′-UTR of the *SUCNR1* gene transcript that affects a binding site for miRNA-4470. We demonstrate that the *SUCNR1* gene and miRNA-4470 are both expressed in the human retina/RPE, and describe that miRNA-4470 binds to the major C allele of

Table 2. SNPs in the 3′-UTR of the *SUCNR1* gene predicted to affect miRNA-binding sites.

SNP	Change in allele	miRNA	Impact of change on miRNA binding
rs56171835	**C ->T**		
		hsa-miR-3655	break
		hsa-miR-4712-5p	enhance
		hsa-miR-578	decrease
		hsa-miR-770-5p	enhance
rs73168929	**A ->C**		
		hsa-miR-3123	create
		hsa-miR-376c	create
		hsa-miR-3976	create
rs73018440	**G ->T**		
		hsa-miR-2116-3p	break
		hsa-miR-4277	enhance
		hsa-miR-4713-5p	break
		hsa-miR-629-3p	break
rs13079080	**C ->T**		
		hsa-miR-4470	break

Impact of the change is indicated as break (no miRNA binding after change), create (no miRNA binding before change), enhance (stronger miRNA binding after change) and decrease (less miRNA binding after change).

rs13079080 leading to reduced mRNA expression, while binding of the miRNA is disrupted by the minor T allele. Moreover, we found a protective association of the TT genotype of rs13079080 with AMD in the EUGENDA cohort, but this association was not confirmed in the IAMDGC dataset.

Interestingly, rs13079080 is located in the 3′-UTR of *SUCNR1* in a region corresponding with the miRNA seed region (Figure 2A), the 5′-end of the miRNA which is crucial for target recognition [22]. More specifically, rs13079080 is the first 5′-nucleotide of the miRNA seed region. The other SNPs found in miRNA-binding sites in the 3′-UTR of *SUCNR1* were either not found in the 5′-end of the miRNA, or the corresponding miRNA was not expressed in RPE cells

in vitro (figure S1). However, that does not mean that the other miRNA's cannot influence the expression of *SUCNR1*. The remaining SNPs could be of interest in other tissues where both the corresponding miRNA and SUCNR1 are expressed. Additional studies are needed to determine the role and consequences of miRNA-4470 on *SUCNR1* regulation. Unfortunately, confirmation of the mRNA degradation effect of miR-4470 on SUCNR1 gene transcript levels in patient derived EBV cell lines did not work out due to low transcript levels. Furthermore, miRNA-4470 is not expressed in murine tissue [28] limiting the options for studying *SUCNR1* regulation by miRNA-4470. As an alternative, iPSC-derived RPE or retinal organoids could be used as a model system to study the effect of the SNP, either using cell lines generated from carriers and non-carriers, or using isogenic lines by introducing the variant by genome editing.

The *Sucnr1* gene is expressed in murine RPE cells [4,9] and recently a link between reduced *Sucnr1* expression and dry AMD was proposed [9]. In that study, *Sucnr1* knockout mice showed signs of sub-retinal accumulations of oxidized-LDL, a component found in drusen. The *Sucnr1* knockout mice also show decreased expression of CD36. CD36 is a scavenger receptor found in RPE cells where it is responsible for the uptake/transport of lipids and oxidized LDL [29]. Downregulation of CD36 (linked to and possibly caused by downregulation of the *Sucnr1*) leads to ineffective removal of these lipids and oxLDL. Eventually, this could lead to drusen formation between RPE cells and Bruch membrane [29] explaining the dry AMD phenotype observed in the mice.

A role for miRNA-4470 mediated *SUCNR1* mRNA reduction in AMD development is supported by expression of miRNA-4470 and *SUCNR1* in human retina tissue. *SUCNR1*
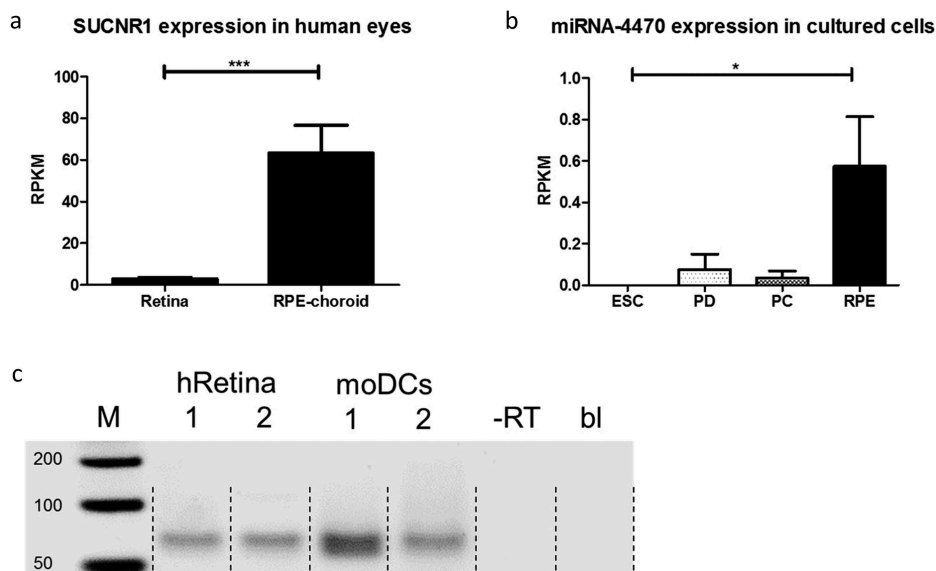

Figure 1. *SUCNR1* and miRNA-4470 are expressed in human eye tissue. A) *SUCNR1* expression (mean + SEM; n = 16) in human retina and RPE-choroid based on RNA-seq data from Kim et al., 2018 [30]. ***, P < 0.001 by student T-test. B) MiRNA-4470 expression (mean + SEM; n = 3) at four different time points of differentiation from hESC towards RPE cells based on RNA-seq data from Hu et al., 2012 [24]. *, P < 0.05 by one-way anova. C) Two primary human retina (hRetina) samples were subjected to an RT-nested PCR reaction to amplify miRNA-4470. Amplified miRNA-4470 (59 bp) was visualized on agarose gel. M = marker showing bands of 50, 100 and 200 bp. – RT is the reverse transcriptase control and bl is the blanco control for the PCR reaction. RPKM, reads per kilobase mapped; ESC, embryonic stem cells; PD, partially differentiated ESC; PC, pigmented clusters; moDCs = monocyte derived dendritic cells.
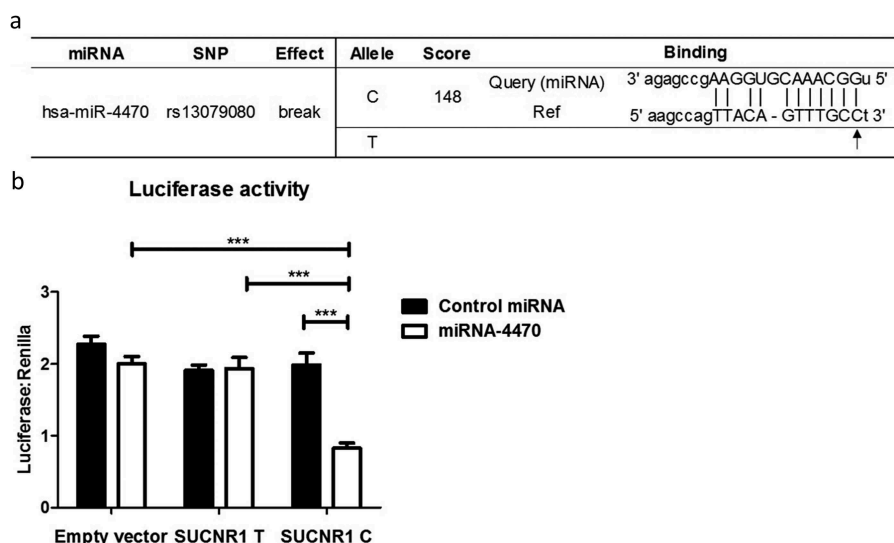
Figure 2. The minor allele of rs13079080 (T) disrupts a miRNA-binding site for and is regulated *in vitro* by miRNA-4470. A) *In silico* analysis of SNPs found in 3′-UTR of *SUCNR1* using mirSNP database [25] indicated an efficient binding of miRNA-4470 with the major C allele, but not the minor T allele, of rs13079080, indicated with an arrow. Score: The predicted score of miRNA-mRNA binding by miRanda software [33]. B) Luciferase reporter constructs carrying the *SUCNR1* 3′-UTR segment of the major (C) or minor (T) allele of rs13079080 (indicated) or without additional segment (empty vector) were transiently-transfected into HEK293 cells together with a miRNA mimic 4470 (white bars) or scrambled miRNA (negative control, black bars). Only in cells transfected with the *SUCNR1* C construct in conjunction with miRNA-4470 a significant decrease in luciferase activity was detected. Data represents mean + SEM (n = 3). ***, P < 0.001 by 2-way anova.

Table 3. EUGENDA cohort and association of rs13079080 with AMD.

	AMD	No AMD	P-value	OR	95% (CI)
N	936	848			
Age (years) – mean (SD)	77.58 (7.57)	72.22 (6.20)	1.95×10^{-45}	1.12	1.10–1.14
Female sex – n (%)	569 (60.8)	481 (56.7)	0.251	1.19	0.97–1.45
SNP rs13079080			0.008		
CC – n (%)	353 (37.7)	305 (36.0)			Reference
CT – n (%)	456 (48.7)	379 (44.7)	0.780	1.03	0.83–1.26
TT – n (%)	127 (13.6)	164 (19.3)	0.007	0.66	0.49–0.89

SD = standard deviation, n = number, OR = odds ratio, CI = confidence interval. Association of rs13079080 with all stages AMD was assessed using a logistic regression model adjusted for age and sex.

is known to be expressed in human RPE-choroid tissue [30] (Figure 1A) but miRNA-4470 has so far only been reported to be expressed in human immune [26] and microglia cells [31]. In this study we demonstrate that miRNA-4470 is also expressed in primary human eye material (Figure 1C).

In this study we identified an association of the rs13079080 SNP in the EUGENDA AMD case-control cohort, in which the TT genotype is associated with a decreased risk for AMD when compared to the CC genotype. No association with AMD was observed for the heterozygous CT genotype (Table 3). Our luciferase assay showed a significant reduction in luciferase activity for the major C allele of rs13079080 compared to the minor T allele (Figure 2B), suggesting that a downregulation of *SUCNR1* expression could be associated with AMD. A downregulation of *SUCNR1* expression in AMD would be in agreement with the observed AMD-like features in *Sucnr1* knockout mice [9].

However, the association between rs13079080 and (advanced) AMD was not confirmed in the IAMDGC dataset (table S1) [27]. The association of rs13079080 in the EUGENDA cohort might represent a false positive finding, or could be related to other factors not accounted for in the

analyses. The association analysis in the EUGENDA cohort was performed on the genotype level and only the homozygous group showed an association (Table 3), which suggests a recessive effect. The AMD GWAS assumed an additive model, which may have failed to detect the effect of the homozygous alternative genotype [27]. Furthermore, the EUGENDA cohort included AMD patients of all stages whereas the AMD GWAS only included advanced AMD patients. Repeating the analysis for the EUGENDA cohort using only advanced AMD resulted in no significant effect but this could be due to the limited number of patients and controls (p = 0.358, table S2). Finally, another possibility for the difference between the two studies is that the effect observed in the EUGENDA cohort may be cohort-, stage of AMD- or population-specific.

Our study demonstrates that the major C allele of rs13079080 in the 3′-UTR of *SUCNR1* transcript forms a binding site for miRNA-4470, potentially resulting in decreased *SUCNR1* expression and consequently reduced capacity of sensing and dealing with oxidative stress. In individuals carrying the T allele, this binding site is disrupted and therefore it is plausible that those individuals have, on the contrary, an increased capacity of sensing and dealing with stress. It would be worthwhile to assess the relevance of this variant in other oxidative stress-associated disorders of which also a possible role for SUCNR1 has been discussed, such as hypertension, obesity-induced adipose tissue inflammation and DM type-1 [5,7,8]. Further studies are needed to assess the expression of miRNA-4470 in additional tissues to determine the relevance of miRNA-4470 regulated *SUCNR1* expression in other stress-related disorders.

Furthermore, SUCNR1 is a highly interesting drug target for the pharmaceutical industry, as the majority of targeted drugs exert their action via G protein-coupled receptors like

the SUCNR1 [2]. For other diseases in which SUCNR1 may be involved (hypertension, obesity-induced adipose tissue inflammation and DM type-1 [5,7,8]) stratification based on the rs13079080 may improve the effects of such drugs.

Methods

Analysis of common variation in the SUCNR1 gene and miRNA expression analysis

The SNPs with minor allele frequency above 0.01 found in the SUCNR1 gene were extracted from the UCSC human genome browser (https://genome.ucsc.edu/) using the dbSNP151 track (Common SNPS (151), HG38, chr3:151873643–151884619). The 12 SNPs found in the 3′-UTR (untranslated-3) were analysed for miRNA binding sites using the mirSNP database [25].

Evaluation of SUCNR1 and miRNA expression in RPE was assessed by retrieving RNA-sequencing data from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) from Kim et al (GSE99248) [30] and Hu et al (GSE37686) [24], respectively. Statistical analyses were performed using Graphpad Prism 5 software. One-tailed Student's t-test or one-way ANOVA with repeated measures, followed by Bonferroni post hoc test, was used, as indicated in figure legends. P values < 0.05 were considered significant.

Analysis of miRNA-4470 expression

Human retina-RPE-choroid samples were obtained from eyes removed during surgery at the Department of Ophthalmology, Radboudumc, Nijmegen, and stored anonymized at −80°C. Total RNA was isolated using TRizol according to the manufacturers' protocol (Invitrogen, Breda, The Netherlands). E.coli poly(A) polymerase (New England Biolabs, Leiden, The Netherlands) was used to add a poly-A tail to all RNA. In short, 1 µl 10X polyadenylation buffer, 1 µl ATP and 1 µl polymerase was added to 2 µg RNA in 10 µl end volume and incubated at 37°C for 60 min. Following inactivation of the polymerase (10 min at 65°C), 2.5 µl of 100 µM oligo-dT adaptor (5′- GCGAGCACAGAATTAATACGACTCACTATAGGTTTT- TTTTTTTTTAA-3′) was added and incubated at 65°C for 5 min to allow for adaptor binding. After a 2 min incubation on ice, cDNA synthesis was conducted using M-MLV reverse transcriptase according to the manufacturer's protocol and following adjustment to the used volume (Invitrogen, Breda, The Netherlands). For the RT- negative control a sample without RNA was taken along. MiRNA-4470 was subsequently amplified using AmpliTAQ gold master mix (Invitrogen, Breda, The Netherlands) using primer set 1 (forward 5′- TCTGGTTGGCAAACGTGGAAG-3′; reverse 5′- GCGAGCACAGAATTAATACGAC-3′) and primer set 2 (forward 5′- ACGTGGAAGCCGAGAAA-3′; reverse 5′- GCGAGCACAGAATTAATACGAC-3′) in the first and nested PCR reaction, respectively. Nested PCR products were visualized on agarose gels containing ethidium bromide. For sequence verification of the fragments, the DNA fragments were isolated from the agarose (Gel Extraction/PCR Clean-up kit, Thermofisher, Amsterdam,

The Netherlands) according to the manufacturer's protocol and subjected to standard Sanger sequence analysis.

Cloning the 3′-UTR sequences of the SUCNR1 in luciferase constructs

Human kidney RNA was used for reverse transcription using M-MLV reverse transcriptase in combination with random hexamer primers according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). The 3′-UTR of the human SUCNR1 (1049 bp-1534 bp from the transcription start site NM_033050.5) was amplified using a high fidelity DNA polymerase (Phusion, Thermofisher, Amsterdam, The Netherlands) using forward (5′- ATAGCTCGAGATCCCTTACATCCTTTAG CAGAT-3′) and reverse (5′- ATGCGGCCGCTTGCT TAACAGCACAACTTCCAA-3′) primers, flanked by XhoI and NotI restriction sites, respectively (underlined). The PCR product was digested with XhoI and NotI (New England Biolabs, Leiden, The Netherlands) and ligated into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Fitchburg, MA) digested with the same enzymes downstream of the luciferase gene. Ligation was performed using T4 DNA ligase (New England Biolabs Ltd, Leiden, The Netherlands). Sanger sequencing revealed that the cloned 3′-UTR DNA fragment contained the major C allele of rs13079080. The minor T allele was subsequently introduced by site-directed mutagenesis (Stratagene, La Jolla, USA) on the obtained pmirGLO-SUCNR1-C construct, using the following primer sequence: forward 5′- CTGTAAGCCAGTTACAGTTTGCTTTAACTCATAGACAT- CAATCAG-3′ and reverse 5′- CTGATTGATGTCTATGAGTT AAAGCAAACTGTAACTGGCTTACAG-3′. The introduction of the T allele (pmirGLO-SUCNR1-T) was confirmed using Sanger sequencing.

Cell culture and luciferase assay

HEK293 cells were grown in Dulbecco's modified Eagle's medium (Lonza, Leusden, The Netherlands) supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM L-glutamine. Cells were grown at 37°C in a humidity-controlled incubator with 5% (v/v) CO_2. Cells were reseeded in a 24-wells plate before transient transfection. Cells were transfected using 50 ng vector DNA (pmirGLO-empty, pmirGLO-SUCNR1-C or pmirGLO-SUCNR1-T) and 50 pmol miR-4470 mimic (UGGCAAACGUGGAAGCCGAGA) or negative control (UCACAACCUCCUAGAAAGAG UAGA) (Dharmacon, Lafayette, USA)) miRNA in combination with 1 µl lipofectamine 2000 (Invitrogen, Breda, The Netherlands). DNA, vector and miRNA mimics, and lipofectamine were each added to 25 µl Optimem (Thermofisher, Amsterdam, The Netherlands) and incubated for 5 min at room temperature. The lipofectamine/DNA mixture was combined and incubated for 20 min at room temperature before adding to the wells. Two days after transfection, luciferase activity was measured. Renilla luciferase present in the vector was used as an internal control for transfection efficiency. Both firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay (Promega, Fitchburg, MA) according to manufacturer's protocol. To

correct for differences in transfection efficiencies, the shown firefly luciferase activity was normalization for Renilla luciferase activity. Statistical analyses were performed using Graphpad Prism 5 software. Two-way ANOVA with repeated measures, followed by Bonferroni post hoc test, was used, as indicated in figure legends. P values < 0.05 were considered significant.

Association analysis of rs13079080 and AMD

Patients and controls from the European Genetic Database (EUGENDA) aged 65 years or older were selected for this study. The grading of AMD was performed using multimodal image grading according to the standard protocol of the Cologne Image Reading Centre (CIRCL) and by certified graders [32]. Genotyping of the rs13079080 SNP was performed using competitive allele-specific KASP genotyping chemistry (LGC, Hoddesdon, UK). Primers and probes were developed by LGC (Primer Allele-FAM: ATCTGTAAGCCAG TTACAGTTTGCC, Primer Allele-HEX: AATCTGTAAGCC AGTTACAGTTTGCT, Primer Common GACACTCT CTGATTGATGTCTATGAGTTA). Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, New York, USA). To determine the association of the demographic variables and the rs13079080 SNP with AMD, a logistic regression was performed including age and sex. The study was performed in accordance with the tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO), and was approved by the local ethics committees. Written informed consent was obtained from all participants. Results from the GWAS on advanced AMD were provided by the International AMD Genomics Consortium (IAMDGC) [27].

Disclosure statement

The authors report no conflict of interest.

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