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Evaluation of sFLT1 Protein Levels in Human Eyes with the *FLT1* rs9943922 Polymorphism

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Abstract

Purpose—Age-related macular degeneration (AMD) is a devastating disease characterized by central vision impairment in individuals with advanced age. Neovascular AMD is a form of end-stage disease in which choroidal vessel outgrowth occurs beneath the retina. While many hypotheses have been raised as to what triggers the formation of pathologic choroidal neovascular membranes, the exact mechanism for their initiation remains unresolved. Polymorphisms in the *FLT1* gene have previously been associated with neovascular AMD risk, including the rs9943922 SNP. Here, we aimed to determine the association between the high-risk *FLT1* genotype and FLT1 protein levels in human retina or RPE/choroid tissue.

Methods—Retina and RPE/choroid tissue from ten human donor eyes was selected from a collection of eyes genotyped for the rs9943922 SNP. Differences in soluble and membrane bound FLT1 protein levels were assessed for retina versus RPE/choroid donor tissue using ELISA and Western blotting analyses. Genotype-associated changes in FLT1 protein levels were also evaluated.

Results—We found soluble FLT1 levels in the RPE/choroid tissue to be approximately three times higher than that of the retina ($p < 0.001$), while both samples have similar levels of the membrane bound form. When tissue with the rs9943922 SNP was compared with controls, no significant genotypic difference in FLT1 protein levels were observed.

Conclusions—Based on these data, we conclude that the rs9943922 SNP in the *FLT1* gene does not result in a large difference in FLT1 protein levels, regardless of whether it is the soluble or the membrane bound form.

Keywords

FLT1; VEGF receptor; choroid; retina; age-related macular degeneration

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

INTRODUCTION

Age-related macular degeneration (AMD) is a major cause of untreatable vision loss in people over the age of 50 in the Western world¹. While signs of early AMD may go unnoticed in patients, advanced forms of the disease often involve devastating loss of vision in the central field. Wet AMD, also known as neovascular or exudative AMD, is characterized by aberrant vessel growth from the choroid into the sub-retinal pigment epithelium or sub-retinal space. These fragile vessels can leak fluid, exudate, or blood causing blurred vision, and if left untreated, can lead to devastating visual decline^{2,3}. About 10% of AMD eyes will develop choroidal neovascular membranes (CNVMs)⁴. The molecular events leading to the conversion of dry to wet AMD are still being uncovered, but recent studies have implicated diverse pathways including complement activation^{5,6}, ER stress⁷, impaired autophagy⁸, increased angiogenic cytokine production by recruited macrophages^{9,10}, MMP-9 upregulation¹¹, and significant alterations to Bruch's membrane composition¹² as playing a role in this transition.

Vascular endothelial growth factor (VEGF) is known to be an important contributor to neovascular membrane development¹³. As the only treatment option currently available for wet AMD, anti-VEGF agents have been very effective at resolving existing CNVMs and restoring vision lost to fluid in the retina^{14,15,16,17,18}. However, there is some concern that prolonged use of anti-VEGF drugs may lead to choriocapillaris dropout and RPE atrophy^{17,19}.

Members of the VEGF family interact with a series of VEGF receptors. VEGF receptor 1, also known as fms-like tyrosine kinase 1 (FLT1), is a membrane bound tyrosine kinase receptor for VEGF-A, VEGF-B, and placental growth factor (PGF). Upon interaction with one of its ligands, membrane bound FLT1 (mbFLT1) acts to promote angiogenesis through the stimulation of endothelial cell survival and proliferation. Due to alternative splicing of the *FLT1* pre-mRNA, a soluble form of the protein (sFLT1) can be synthesized, which binds to and sequesters VEGF. The alternatively spliced sFLT1 prevents VEGF from activating the canonical membrane bound receptor, impairing its angiogenic function. Therefore, maintaining an appropriate ratio between mbFLT1 and sFLT1 in the eye is important for vascular homeostasis^{5,20}.

In normal physiology, sFLT1 contributes to maintaining an antiangiogenic environment in the outer neural retina. Using mice with a conditional *Flt1* knockout in RPE cells, Luo et al. provided evidence for the importance of sFLT1 in preserving an avascular environment for photoreceptor cells. Importantly, anti-sFLT1 subretinal injections lead to CNV in control mice²¹.

In light of the importance of VEGF signaling in neovascular AMD, Owen and colleagues assessed the association of various *FLT1* SNPs with AMD risk. Out of a large number of SNPs examined, they found rs9943922 showed a significant association with an increased risk of neovascular AMD, with a 1.48-fold increase²². This particular SNP results in a T to C nucleotide substitution within an intronic region of the *FLT1* gene. While the mutation does not affect splicing, in silico analysis indicates it may alter the secondary structure of the

FLT1 RNA molecule²², which could potentially shift the ratio of mbFLT1:sFLT1 toward an increase in the angiogenic form of the receptor. Consistent with this possibility, one recent study showed that sFLT1 serum levels are lower in patients with neovascular AMD, compared with non-AMD patients and those with early AMD. These investigators also reported an association between serum sFLT1 levels <80pg/mL and neovascular AMD in people over 73 years of age, although they did not stratify by genotype²³. When taking genotype into account, in individuals homozygous for the rs9943922 SNP, there are lower levels of sFLT1 in the serum of those with neovascular AMD compared to healthy controls²². In the current study, we sought to determine whether the abundance of *FLT1* is altered in human ocular tissues homozygous for high or low risk genotypes in the *FLT1* gene.

MATERIALS AND METHODS

Donor eye tissue

Ninety-three human donor eyes were selected from a tissue collection obtained from the Iowa Lions Eye Bank (Iowa City, IA) with the families' full informed consent and within 8 hours after death. Eyes were used in accordance with the Declaration of Helsinki. After selection of control eyes or those homozygous for the rs9943922 single nucleotide polymorphism, a subset was used for biochemical experiments. For these experiments, a 6-mm punch from the peripheral retina (centered approximately 7mm from the fovea centralis) had been previously collected for each of 10 donors, and the neural retina and RPE/choroid were snap frozen separately for later biochemical processing. Disease affection status was determined for each donor by chart review. While none of the eyes used for biochemical analyses had advanced AMD, two eyes had a clinical diagnosis of "dry AMD" or "mild dry AMD". Histological evaluation of the maculas from these donors did not reveal confluent basal laminar deposits, drusen or gross changes in RPE morphology, and were thus not removed from the study. Additional information for these 10 donors can be found in Table 1.

Genotyping

DNA was prepared from a panel of 93 donors, either from a blood sample or from extraocular muscle tissue using the DNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol²⁴. DNA was genotyped at the *FLT1* rs9943922 single nucleotide polymorphisms (SNP) that was previously associated with increased risk of neovascular AMD using a commercially available real-time PCR assay (TaqMan, Applied Biosystems; Foster City, CA) with an ABI 7900HT(Fast) Real-Time PCR System following the manufacturer's protocol. The 10 donors used for this study were selected from this list.

FLT1 Western blotting

Human retina and RPE/choroid tissue from donors genotyped for the *FLT1* SNP rs9943922 (5 TT and 5 CC donors) was used. 6mm-diameter posterior eye tissue punches were collected, the retina was separated from the RPE/choroid, and total protein was extracted from each sample using a solution of 10mM phosphate buffered saline with protease inhibitors (Complete, Roche) with 1% Triton X-100. Protein concentrations were determined using a BioRad DC Protein Assay. 20µg of protein was diluted 3:1 in 4x

Laemmli Sample Buffer (with 1% β -mercaptoethanol) and boiled for 5 minutes. Proteins were separated on a 4–20% Mini-PROTEAN TGX Gel that included WesternC molecular weight standards (BioRad, Hercules, CA) and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 1% BSA in 1x PBS with 1% Tween-20, and subsequently incubated with monoclonal rabbit anti-VEGFR1 (FLT1) antibody (ab32152, Abcam, Cambridge, MA), followed by Alexa Fluor 488 donkey anti-rabbit IgG antibody (A-21206, Molecular Probes, Eugene, OR). The blot was imaged using a VersaDoc imager (unless otherwise specified, all reagents and equipment were obtained from BioRad, Hercules, CA).

sFLT1 ELISA

Human donor retina and RPE/choroid tissue was used. Five donors homozygous for the *FLT1* SNP rs9943922 (CC) were compared to five donors homozygous for the T allele. 6mm-diameter posterior eye tissue punches were collected from the peripheral tissue for each donor, and the retina was separated from the RPE/choroid. Total protein was extracted from each sample as described above. Protein concentrations were determined using a BioRad DC Protein Assay (BioRad). 22 μ g and 28 μ g of total protein for retina and RPE samples, respectively, were loaded into each well of a sFLT1 ELISA kit (sVEGFR1, Boster Biological Technology) in duplicate, according to the manufacturer's instructions. Standards provided by the kit were used to determine sFLT1 concentration in each sample according to absorbance values and standard curve linear regression line. The ELISA data was analyzed for significance using a two-tailed Student's t-test, with data points exhibiting an uncorrected p-value < 0.05 being considered significant.

RESULTS

Genotyping

Genotypes at the *FLT1* SNP associated with AMD, rs9943922, were obtained for a panel of 93 human donor eyes. Of those samples genotyped at SNP rs9943922, twenty-two samples were homozygous for the high-risk allele, thirty-six were homozygous for the low-risk allele, and the remainder were heterozygotes. Five samples of unfixed retina and RPE/choroid were identified from the 22 donors homozygous for the high-risk SNP rs9943922, and five samples of unfixed retina and RPE/choroid were identified from the 36 donors homozygous for the low-risk SNP. These samples were employed in subsequent analyses (the remainder were from aldehyde fixed eyes or were otherwise unsuitable for biochemical study), and additional donor information can be found in Table 1.

sFLT1 ELISA

Levels of sFLT1 were quantified in donor retina and RPE/choroid tissues. Individual donors showed considerable variability, with concentrations ranging from <1 to 250pg/mL in retina and about 80 to 600pg/mL in RPE/choroid. While considerable variability was observed between donors, average sFLT1 concentration did not significantly differ between genotypes (Figure 1). When comparing sFLT1 levels in the retina to sFLT1 levels in the RPE/choroid, we found RPE/choroid tissue to have significantly more sFLT1 levels (data not shown, $p < 0.001$).

FLT1 Western blot

While ELISA can accurately determine abundance of a protein, it does not allow us to visualize differences in protein isoforms. We therefore evaluated genotyped retina and RPE/choroid samples using Western blotting. While we did not observe a band for the canonical 77kDa sFLT1 protein (the retina blot shows a non-specific band around 90kDa, which disappeared when the β -actin antibody was omitted from the blot), a band was present at ~60kDa, which matches the predicted molecular weight of isoform 4 of the FLT1 protein (REFSEQ ID NM_001160031.1). A significant difference in this species or of mbFLT1 protein levels was not observed based on genotype alone. However, there was a trend toward more mbFLT1 in the retina for CC donors compared to TT donors. A difference in levels of FLT1 (60 kDa) and mbFLT1 in retina versus choroid was readily apparent (Figure 2). Retina tissue from all donors had similar mbFLT1 levels with little or no sFLT1 present (Figure 2A). In contrast to what was observed for retina, there was a 60 kDa band present for FLT1 in the RPE/choroid samples for all donors (Figure 2B). There was some slight variability in amount of FLT1 between donors, but this variability was not attributable to genotype.

DISCUSSION

To date, variations in 34 loci have been reported to contribute to AMD with genome wide significance²⁵, including the most prominently associated polymorphisms in the *CFH* and *ARMS2/HTRA1* loci. However, while these variants predispose individuals to all forms of AMD, few genetic variants have been described as participating in wet AMD selectively^{25,26}. One such variant is a short tandem repeat polymorphism in *MMP9*²⁵, which was not assessed in this study, and others include polymorphisms in the *FLT1* gene²². Owen et al. suggest an association between *FLT1* SNPs and increased risk of wet AMD development within four cohorts²². In the current study we sought to determine if the high-risk alleles in the study by Owen et al. result in altered levels of the encoded FLT1 protein.

Based on our biochemical analyses, sFLT1 levels are not significantly different between genotypes tested, but there is variation between choroid and retina. The current study has a number of limitations. It is possible that our modest sample size (5 per category) was not sufficient to detect a subtle difference in FLT1 abundance. We estimate that for RPE-choroid, our sample has over 80% power to detect a 50% increase with 5% probability of a type I error (<https://www.dssresearch.com/knowledgecenter/toolkitcalculators/statisticalpowercalculators.aspx>). Moreover, as our biochemical samples were collected just outside the arcade vessels, if FLT1 regulation is different in the macular than peripheral retina/choroid, we would not be able to detect this difference. In addition, our study evaluated total protein levels, not new synthesis, and the detected sFLT1 could be higher in choroid (if present in blood) and lower in retina (if secreted into vitreous) than would be predicted by RNA synthesis. Thus, while we cannot rule out a contribution to the overall protein abundance due to the presence of the rs9943922 SNP, it is apparent that the SNP is not sufficient to alter protein abundance with the current sample size. It is possible, however, that a small variation may be observed if more samples are added to this study. Despite the lack of altered protein levels observed in our analyses, the rs9943922 SNP may impact the kinetics of VEGFR1 synthesis and folding, as suggested for HTRA1²⁷, with a corresponding

impact on VEGF binding and receptor activation in the choroid and/or retina. This type of change could shift the mbFLT1:sFLT1 ratio (based on function, not protein levels) toward an angiogenic phenotype. Future work will be needed to assess the extent of sFLT1 binding to VEGF in relation to the AMD-associated SNPs.

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Figure 1. ELISA quantification of sFLT1 levels based on genotype

sFLT1 concentration is unchanged in donors with rs9943922 *FLT1* CC genotype compared to those with the TT genotype. **A.** Average retina sFLT1 level for each genotype is indicated.

B. Average RPE/choroid sFLT1 level for each genotype is indicated. Error bars indicate standard error of the mean.

Figure 2. Evaluation of FLT1 isoforms by Western blot

A. Lanes 1 and 12 contain WesternC standard. Lanes 2-6 represent the TT donors while lanes 7-11 represent the CC donors. **B.** Lanes 1 and 12 contain WesternC standard. Lanes 2-6 represent the TT donors while lanes 7-11 represent the CC donors. mbFLT1 and FLT1 (isoform 4) were observed at 160 kDa and 60 kDa, respectively. The 40 kDa bands represent β-actin.

Table 1

Table of donor information.

Donor	FLT1 genotype	Age	Sex	sFLT1 ELISA Concentration		
				Retina	RPE/Choroid	
1	TT	76	M	374 pg/mL	1774 pg/mL	
2	TT	72	F	0 pg/mL	914 pg/mL	
3	TT	62	F	0 pg/mL	504 pg/mL	
4	TT	70	M	194 pg/mL	1354 pg/mL	
5	TT	76	M	0 pg/mL	734 pg/mL	
6	CC	82	F	0 pg/mL	1304 pg/mL	
7	CC	73	M	0 pg/mL	1254 pg/mL	
8	CC	64	F	814 pg/mL	744 pg/mL	
9	CC	79	M	0 pg/mL	644 pg/mL	
10	CC	72	M	0 pg/mL	574 pg/mL	