Gene 523 (2013) 106–111

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short communication

CC chemokine receptor-3 as new target for age-related macular degeneration

Neel Kamal Sharma [a], Amod Gupta [b], Sudesh Prabhakar [a], Ramandeep Singh [b], Arvind Kumar Bhatt [c], Akshay Anand [a,*]

[a] Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India
[b] Department of Ophthalmology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India
[c] Department of Biotechnology, Himachal Pradesh University, Shimla, India

ARTICLE INFO

Article history:
Accepted 6 March 2013
Available online 6 April 2013

Keywords:
CCR3
Inflammation
Chemokines
Single nucleotide polymorphism
Population study
AMD

ABSTRACT

CC chemokine receptor-3 (CCR3) is involved in angiogenic processes. Recently, CCR3 was accounted to participate in choroidal neovascularization (CNV) and CCR3 targeting was reported to be superior to standard antivascular endothelial growth factor-A (VEGF-A) administration when tested in an artificially induced CNV in animals. As human CCR3 studies are lacking in age-related macular degeneration (AMD) patients we sought to determine if CCR3 has any association with inflammatory processes that occur in CNV. A total of 176 subjects were included on the basis of inclusion criteria. Real time PCR was used to analyze the single nucleotide polymorphism in CCR3 of AMD (115) and normal controls (n = 61). Genotype frequency was adjusted for possible confounders like cigarette smoking, alcohol, meat consumption and other risk factors. Chi-square test was used for analysis of polymorphism. The genotype distribution of CCR3 (rs3091250) polymorphism was significantly different in AMD patients in the Indian population. GT (heterozygous) and TT (homozygous) at the rs3091250 SNP increased risk of AMD as compared to the GG genotypes (OR = 4.8, CI 95% = 2.2–10.8 and OR = 4.1, CI 95% = 1.6–10.1 respectively). Subgroup analysis of AMD patients in wet and dry revealed no significant differences. There was no significant difference for rs3091312 in AMD and control group. A significant association between AMD and CCR3 (rs3091250) polymorphism localized on chromosome 3p21.3 was detected. The results suggest the possible contribution of rs3091250, a new predisposing allele in AMD.

1. Introduction

Age-related macular degeneration (AMD) is a primary cause of central vision loss in the aged in industrialized countries (Cook et al., 2008). Symptoms of AMD may appear in one or in both eyes. Early symptoms include metamorphopsia or blurring of central vision. AMD is characterized by the development of drusen in Bruch's membrane, the degeneration of the macular retinal pigment epithelium (RPE), geographic atrophy and neovascularization.

According to clinical age-related maculopathy grading system, age-related maculopathy grades are: without drusen, several minute drusen and no RPE changes, retinal RPE alteration but no drusen, both small drusen and RPE changes, several large and intermediate-size drusen, RPE detachment, geographic atrophy and choroidal neovascular membrane with disciform scarring (Seddon et al., 2006).

It is established that there is a complex participation of environmental and genetic factors in the pathogenesis of AMD. In complement system, microglial recruitment, inflammation, DNA repair, and neovascularization activation studies have identified numerous AMD-associated genes (Ding et al., 2009).

A lot of the earlier works done on genetic factors impacting AMD have been focused on single nucleotide polymorphisms (SNPs). Although extremely significant statistical relations between various single nucleotide polymorphisms and AMD have been discovered, they do not account for the whole genetic aspect of the disease. It is supposed that complement activation resulting from dysfunction of CC chemokines may contribute to inflammation. The infiltration of monocytes is accompanied by inflammatory chemokines as key mediators.

Studies recently indicated that inflammation plays a fundamental role in the development of CNV (Rohrer et al., 2009). Additionally, genetic evidence has identified variations in multiple genes (Sharma et al., 2009). Studies had also investigated the role of asthma with AMD and found that asthma could be a risk factor for AMD (Sun et al., 2012).

In senescent mice deficient in monocyte chemoattractant protein-1 (CCL2, also known as MCP-1) or its receptor we earlier described the

Abbreviations: AMD, age-related macular degeneration; CCR3, CC chemokine receptor-3; CEC, choroidal endothelial cell; CNV, choroidal neovascularization; FFA, fluorescein fundus angiography; GPCR, G-protein-coupled receptor; MCP1, monocyte chemoattractant protein-1; OCT, optical coherence tomography; RPE, retinal pigment epithelium; SDS, Sequence Detection System; SNPs, single nucleotide polymorphisms; VEGF-A, vascular endothelial growth factor-A.

* Corresponding author. Tel.: +91 9914209090, +91 1722756094; fax: +91 1722748399.
E-mail address: akshay1anand@rediffmail.com (A. Anand).*URL:* http://www.neurologypgi.org (A. Anand).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.03.052

spontaneous development of CNV postulating its key role in AMD pathogenesis (Ambati et al., 2003). We hypothesized that CCR3 is also involved in similar processes and any polymorphism in this gene may consequence in chronic inflammation by continued activation of the complement system contributing to the pathogenesis of AMD.

Antivascular endothelial growth factor treatment is currently used for wet AMD patients (The CATT Research Group, 2011). Even though Eghøj and Sorensen (2012) showed that out of the 1076 eyes, a total of 20 (2%) eyes met the criteria for tachyphylaxis i.e. drug did not respond at the time of reactivation of CNV in AMD patients who had responded to the treatment initially anti-VEGF-A therapy produces dysfunction and damage to the outer and inner murine retina (Nishijima et al., 2007; Saint-Geniez et al., 2008), raising a question of recurrent CNV or potential retinal toxicity. In this context it is pertinent to review the report of Takeda et al. who reported that the G-protein-coupled receptor (GPCR), CCR3 is important in neovascular AMD showing that CCR3 neutralizing antibodies are more effective than VEGF-A neutralizing antibodies in inhibiting the CNV in mice model (Takeda et al., 2009). Furthermore, genetically engineered mice that were lacking in CCR3 or its ligands were also protected to some extent from the effect of laser injury on the choroidal vasculature (Takeda et al., 2009). Additional evidence also supports its role in CNV (Ahmad et al., 2011). However, a study recently showed that CCR3 was not significant in CNV development when using a Matrigel CNV model (Li et al., 2011). Therefore, further study regarding the potential role of CCR3 in AMD is needed.

CCR3 is a receptor for eotaxin found on the surface of a variety of cells, including white blood cells. It is most commonly related with mast cells and eosinophils that play a main role in allergic reactions (Pope et al., 2005) as well as angiogenesis (Takeda et al., 2009). CCR3 gene is located on the short arm of chromosome 3. AMD is a complex disease, which is influenced by genetic and environmental factors. The absence of any such genetic association studies of CCR3 and AMD prompted us to explore the role of this chemokine in these patients. We therefore wanted to determine the polymorphism of CCR3 in the human AMD patients.

2. Materials and methods

The study population included 176 subjects, which include 115 AMD patients and 61 normal healthy controls from the Advanced Eye Center, Post-Graduate Institute of Medical Education and Research, Chandigarh, India. 50 years or older AMD patients with the diagnosis of advanced AMD including geographic atrophy and/or choroidal neovascularization with drusen more than five in at least one eye were incorporated in the study. The controls in the study included those more than 50 years of age with the absence of other diagnostic criteria for AMD.

The exclusion criteria defined retinal diseases involving the photoreceptors and/or outer retinal layers other than AMD loss such as high myopia, central serous retinopathy, retinal dystrophies, diabetic retinopathy, vein occlusion, uveitis or similar outer retinal diseases that have been present before an age of 50 and opacities of the ocular media, limitations of pupillary dilation or other problems enough to preclude sufficient stereo fundus photography. These conditions include cataracts, opacities due to ocular diseases and occluded pupils due to synechiae. Ethical approval was taken for the study by the Institute Ethics Committee, Post-Graduate Institute of Medical Education and Research, Chandigarh, India vide letter No Micro/10/1411. Informed consent was taken in the approved format endorsed by the Institute Ethical Committee.

2.1. Ophthalmic examination

Patients underwent complete clinical ophthalmic examination by a retina specialist for best corrected visual acuity, slit lamp biomicroscopy of anterior segment and dilated fundus examination. All AMD patients were subjected to fluorescein fundus angiography (FFA) and optical coherence tomography (OCT). The diagnosis of AMD was based on ophthalmoscopic and FFA findings.

2.2. Demographic characterization

All the subjects were informed of the purpose of the study and interviewed. A written informed consent was taken from individual participants. The risk factor questionnaire included information about demographic characteristics such as cigarette smoking, alcohol intake, food habit, comorbidity etc. Smokers were defined as those having smoked at least three cigarettes per day or 54 boxes for at least 6 months and were segregated further into smokers and never smokers. Non-vegetarian patients were defined as those having chicken, meat or fish for at least 6 months and alcoholic patients were defined as those having whiskey, rum, wine or homemade alcohol for at least 6 months. Co-morbidity was determined based on the participant's responses to whether any physician had told them for diagnosis of stroke, migraine or any heart diseases. Subjects were also asked to report any prior diagnosis of any neurological, cardiovascular or metabolic disorders etc.

2.3. Selection of single-nucleotide polymorphisms

The SNPs used in our study have been previously examined for other allergic and inflammatory diseases like asthma, because like CNV, asthma is a multifactorial disorder with both genetic and environmental factors (Mizutani et al., 2009). Because some of the mechanisms of progression of both CNV and asthma are similar (Sun et al., 2012), we hypothesized that there is an association between these diseases. Previously several population-based studies have accounted asthma to be associated with elevated risk of developing CNV.

2.4. DNA isolation

DNA was extracted using DNA extraction kits (Qiagen) as per the instructions provided by the manufacturer. Extracted DNA was stored at 4 °C to further investigate the polymorphism in CCR3 gene.

2.5. Real time PCR

Real time PCR was used to analyze SNPs and was performed in the 48 well model StepOne™ (Applied Biosystems Inc., Foster city, CA) using published TaqMan® SNP Genotyping Assays. Real time PCR was carried out for 20.0 μl volume containing 10 μl master mix, 5 μl Assay (Applied Biosystems) and 20 ng DNA was added to make the volume 20.0 μl. TaqMan® SNP Genotyping Assays (Applied Biosystems) was used to carry out all reactions according to the manufacturer's recommendations. Two reporter dyes FAM and VIC were used to label the Allele 1 and 2 probes and 5' Nuclease Assay was carried out. Negative controls in the PCR mix did not contain DNA. PCR amplification and SNP estimation were done by StepOne TM v 2.0 software (Applied Biosystems Inc., Foster City, CA). Sequence Detection System (SDS) software was used to import the fluorescence measurements made during the plate read to plot fluorescence (Rn) values after PCR amplification.

2.6. Statistical analysis

Genotypes estimated by the real time PCR for each mutation were stratified for homozygosity and heterozygosity for the respective allelic variants. Between various groups association was analyzed by Pearson's chi-square test. Genotype distributions were analyzed by logistic regression, integrating adjustments. Genotypic associations and odds ratios (ORs) with 95% confidence intervals (CI) were estimated by binary logistic regression. The $p \leq 0.05$ was considered to be significant. SPSS 20.0 software was used to perform statistical analysis.