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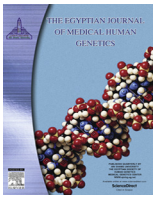

Original article

# Copy number variation in *VEGF* gene as a biomarker of susceptibility to age-related macular degeneration

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ABSTRACT

*Background:* Several studies in various populations have been conducted to determine candidate genes that could contribute to age-related macular degeneration (AMD) pathogenesis.
*Objective:* The present study was undertaken to determine the association of high temperature requirement A-1 (*HTRA1*), vascular endothelial growth factor (*VEGF*) and very-low-density receptor (*VLDR*) genes with wet AMD subjects in Malaysia.
*Methods:* A total of 125 subjects with wet AMD and 120 subjects without AMD from the Malaysian population were selected for this study. Genomic DNA was extracted and copy number variations (CNVs) were determined using quantitative real-time Polymerase Chain Reaction (qPCR) and comparison between the two groups was done. The demographic characteristics were also recorded. Statistical analysis was carried out using software where a level of $P < 0.05$ was considered to be statistically significant.
*Result:* Statistically significant associations of the *VEGF* gene were observed in mean copy differences between case and control subjects ($P < 0.05$). The consistency of both unadjusted and age-adjusted data at Copy Number CN gain (CN = 3 and CN = 4) suggested that *VEGF* could increase the risk of wet AMD disease ($P < 0.05$). None of CNVs of *HTRA1* and *VLDR* genes showed associations with the wet AMD disease based on comparisons of the frequencies of mean ($P > 0.05$).
*Conclusion:* Observations of an association between CNVs of *VEGF* gene and wet AMD have revealed that the CNVs of *VEGF* gene appears to be a possible contributor to wet AMD subjects in Malaysia.


## 1. Introduction

Age-related macular degeneration (AMD) is a common form of irreversible vision loss and a leading cause of blindness among the elderly [1]. The prevalence of AMD among Japanese (0.87%) [2], Chinese (0.2%) [3], Taiwanese (1.9%) [4], Singaporean Malays (0.34%) [5] and Indians (1.9%) [6] are expected to increase with the increasing aging population.

Neovascular or wet AMD is a complex multifactorial disease associated with environmental and genetic risk-factors in many populations. Wet AMD is characterized by abnormal blood vessel (choroidal neovascular membrane) growth and leakage in the choroid that results in subretinal bleeding and scar formation [7]. Despite intensive research efforts, the genetic factors for the development of nAMD remain unclear. Genetic variants found in interleukin, the chemokine (C-C motif) ligand 2 (*CCL2*) and the chemokine (C-X3-C motif) ligand 1 (*CX3CL1*) genes are likely to be responsible for the development of AMD among various ethnicities. *CCL2* and *CX3CL1* genes appear to be crucial in monocyte and microglial cell recruitment to the sub-retinal space in AMD [8,9]. Most of the studies revealed highly significant associations of

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various Single Nucleotide Polymorphism (SNPs) and wet AMD [10]. However, recently copy number variations (CNVs) are also linked to various diseases such as systemic lupus erythematosus [11], hypertension-left ventricular hypertrophy [12], and rheumatoid arthritis [13]. CNVs may account for a significant proportion for the development of various eye diseases in many population studies with conflicting results (Table 1).

PCR-based arrays are commonly used to detect CNVs, in particular quantitative PCR (Q-PCR) are used to determine CNVs precisely for specific candidate genes. Q-PCR is considered as a most reliable and cost effective method compared to conventional PCR and Multiplex ligation dependent probe (MLPA) [12]. The occurrences of CNVs in genes are tending to be highly influential for the individuals predicted to be at high risk for the development of wet AMD. Candidate genes such as Complement Factor H (*CFH*), vascular endothelial growth factor (*VEGF*), high temperature requirement A-1 (*HTRA1*) and very-low-density receptor (*VLDR*) with their putative functions are known to be responsible for the development of wet AMD [14].

In the present study, we analysed the polymorphisms of the three candidate genes; *VEGF, HTRA1* and *VLDR* genes which are identified to be associated with both risk of, and protection against, AMD [15,16]. To our knowledge, there are lacks of report on CNVs among wet AMD subjects particularly in Malaysia. This led us to determine whether the CNVs in *VEGF, HTRA1* and *VLDR* genes could be possible contributor for the development of wet AMD or not.

## 2. Subjects and methods

### 2.1. Study subjects

Based on clinical findings in this study, we recruited 245 subjects [125 wet AMD and 120 without AMD cases] using an inclusion and exclusion criteria as a basis for selection. Sample size was adequate for the present study, which is a minimum sample of 110 in each group was calculated based on the ratio of 1:1 for case and control groups, was at significance level of $P < 0.05$ at power 80% on the basis of prevalence of minor alleles referred from previous studies [5,17].

Subjects who undergone comprehensive ophthalmic examinations and diagnosed as wet AMD and presence of choroidal neovascularization (CNV) in either or both eyes, were included in this study [18]. The three main ethnicities (Malay, Chinese and Indians), both male and female subjects, were also included in this study. Polypoidal choroidal vasculopathy and the other retinal disorders were excluded by indocyanine green angiography (ICG) method. Control subjects were free of any eye disorders at the time of ascertainment. They were screened for a complete eye examination by the ophthalmologist and/or review of eye clinic charts. Those with significant signs of retinal diseases such as central serous retinopathy, myopia, retinal dystrophies, diabetic retinopathy, vein occlusion, uveitis and dry AMD were excluded from the control group. A questionnaire was prepared in both Malay and English languages and were distributed to all the subjects to assess their demographic data, relevant medical history and smoking history. Both male and female subjects were recruited from the three main ethnic groups: Malay, Chinese and Indian under this study. A total of 5 ml of peripheral blood were drawn by phlebotomists into an EDTA tubes for further analysis. Genomic DNA from peripheral blood was isolated with QIAamp Blood DNA Mini Kit (QIAGEN, Germany). The purity of extracted DNA was quantified by Nanodrop ND-1000 spectrophotometer (Nano-drop Technologies, Wilmington, DE) and qualified using gel electrophoresis. Amplification and annealing temperatures of the PCR products were optimized using thermal cyclers (Thermo Fisher Scientific, Finland) as in Table 2.

In this study, the qPCR method was used to determine the genetic CNVs in VEGF, HTRA1 and VLDR genes. QPCR was performed with the MiniOpticon Real-Time PCR System (Bio-rad Hungary Ltd) based on the following thermal cycling conditions: holding at 95 °C for 1.0 min, 40 cycles for 5 s at 95 °C for denaturation and 20–30 s at 60–65 °C for the amplification of all genes. Individual real-time PCR reactions were carried out in 20 μl volumes in a 48-well plate containing 1.0 uL of genomic DNA, 10.0 uL 2X qPCR BIO SyGreen Mix L0-Rox Master Mix, 0.8 uL of each primer and run simultaneously with a Type-it CNV Reference Primer Assay according to manufacturer's instructions.

TERT (telomerase reverse transcriptase) is a single copy gene that is used as endogenous reference genes for qPCR-based CNVs validation [12]. However, in our study 10% of samples were selected randomly and the assay was carried out on two separate occasions and the results were consistent and identical. CNVs gain is defined as CN higher than 2 (>2 copies), and CN lower than 2 (<2 copies) would be regarded as CNVs loss [19]. In the qPCR assay, the cycle threshold (CT) value is defined as the number of cycles required for the fluorescent signal to cross the threshold exceeds background level. The CT values was generated from equivalent standard curve mass points (target versus reference) which used in the ΔCT calculation (CT target – CT reference, normalized by relative quantitative PCR (ΔΔCT) then followed by CNVs ratio formula calculation ($2 - \Delta\Delta Ct$) with their sensitivity is commonly used to validate the CNVs in association studies [20,21].

### 2.2. Statistical method

All statistical and analysis data were secured using statistical software (IBM SPSS Statistics version 22, US). Logistic regressions, t-tests and age-adjusted analysis of covariance were performed to compare the frequencies of each copy number category (CN = 0, 1, 2, or 3+) and the mean number of copies of each gene, respectively, between the AMD subjects and controls. All numerical variables that were evaluated show normally distributed after the Kolmogorov-Smirnov test analysis.

Logistic regression was used to compare the odds of wet AMD among the various copy number levels. Analysis of covariance was performed to compare the mean copy numbers for both categorical and continuous covariates. Statistical significance was deemed to have been attained for all analysis when the p-value was lower than 0.05 ($P < 0.05$).

### Ethical approval

Ethical approval was obtained from the Ethics Committee of Universiti Kebangsaan Malaysia (Reference: FF-2014-206) and the University Ethics Committee for Research Involving Human Subjects (Reference: MREC15, P008) under the Putra Grant, UPM Project (No. 9409800) followed by the National Medical Research Register (Reference: NMRR-14-1176-21475). This work also has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in human. An informed consent was obtained from all the subjects.

## 3. Results

### 3.1. Analysis of CNVS

Fig. 1 shows the amplification plots for quantitative copy numbers which were determined using differences in the amplification