Study protocol and Statistical analysis plan
#NCT02707575

Cytokeratin 8 level in Aqueous Humor, as a prognostic factor for visual and anatomical outcomes after Ranibizumab (Lucentis) in Neovascular Age-related Macular Degeneration

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1. Objectives:

The cytokeratin 8 (CK8) level of aqueous humor in neovascular age-related macular degeneration (nAMD) patient is related to the visual and anatomical outcomes after Lucentis (Ranibizumab).

Primary objective To investigate the correlation between the CK8 level of aqueous humor in neovascular agerelated macular degeneration (nAMD) patient and the best corrected visual acuity change after Lucentis

Secondary objectives To investigate the correlation between the CK8 level of aqueous humor in neovascular agerelated macular degeneration (nAMD) patient and the retinal thickness change after Lucentis (Ranibizumab).

Explorative objectives: Associations of the baseline CK8 level with patients characteristics (with age, sex, type of new vessel (NV) and the OCT parameters including CNV size and central subfield macular thickness (CMT).

2. Design

non-randomized, open-label, Prospective observational study

3. Method

The prospective study was performed at the Severance Hospital, Gangnam Severance Hospital of

Yonsei University and Isan Paik Hospital of Inje University between April 2016 and April 2018.

Procedures were in accordance with the Declaration of Helsinki and approved by the ethics committee

of each institution. All study participants provided written informed consent.

Study participants

All patients were required to be least 50 years old with newly diagnosed (treatment-naïve) nAMD. The disease must be recent and confirmed via medical history and clinical findings. Patients were excluded if their eyes exhibited one of the following features: myopia with a refractive error greater than ±3.0 diopters or evidence of pathologic myopia (preoperative refractive data were used to assess pseudophakic eyes); any history of vitrectomy, anti-VEGF therapy, laser treatment, or photodynamic therapy (PDT); history of cataract surgery within 3 months prior to presentation; evidence of end-stage AMD such as subfoveal fibrosis or atrophy; eyes with large submacular hemorrhage (SMH) over 1 disc-diameter; evidence of other retinal diseases including central serous chorioretinopathy, diabetic retinopathy, hypertensive retinopathy, or other neovascular maculopathies; glaucoma; poor imaging data due to media opacity; and unstable fixation. Patients with uncontrolled systemic diseases, use of immunosuppressive drugs, or malignant tumors at any location were also excluded. Controls were age- and sex-matched patients who underwent cataract surgery. Preoperative evaluation excluded individuals from the control group if they had eyes with ophthalmic diseases other than cataracts or eyes that met the exclusion criteria.

Baseline evaluation, treatment, and AH sampling

At baseline, each patient in the nAMD group underwent a comprehensive ophthalmological examination assessing best-corrected visual acuity (BCVA), intraocular pressure (IOP), autorefractometry/keratometry, slit-lamp biomicroscopy, indirect ophthalmoscopy, color fundus photography (FP), fluorescein angiography (FA), indocyanine green angiography (ICGA) (Optos® P200Tx, *Optos* PLC, Dunfermline, United Kingdom), and OCT (Swept source OCT DRI OCT Triton, Topcon, Tokyo, Japan). After baseline evaluation, nAMD patients were subjected to three consecutive intravitreal injections of 0.5 mg ranibizumab (Lucentis; Novartis, Basel, Switzerland) for 3 months, using a standardized protocol. Treatment outcome was monitored (BCVA, IOP, slit-lamp biomicroscopy, FP, and OCT) every time patients received an injection (baseline, Month 1, Month 2) and at 1 month after the third injection (Month 3). Along with injections, AH samples were taken at baseline, Month 1, and Month 2. Preoperatively, the eye was anesthetized topically using 0.5%

proparacaine hydrochloride. A 10% povidone-iodine scrub of lids and lashes was performed, and 5% povidone-iodine eye drops were applied to the ocular surface. The eye was draped in a sterile fashion, and a sterile lid speculum was inserted. A mean volume of 0.1 mL AH was collected via anterior chamber limbal paracentesis using a 30-gauge needle attached to an insulin syringe. Next, patients were given 0.5 mg IVR through the pars plana, 3–3.5 mm from the limbus. They were postoperatively treated with antibiotic eye drops (0.5% moxifloxacin hydrochloride) for 3 d.

Control subjects also underwent a comprehensive preoperative ophthalmological examination, including BCVA, IOP, ARK, slit-lamp biomicroscopy, indirect ophthalmoscopy, FP and OCT. Their AH samples were obtained immediately before cataract surgery.

Measurement of CYTOKERATIN 8 in AH

Immediately after collection, AH samples were transferred to sterile plastic tubes (safe-lock microcentrifuge tubes, 1.5 mL), immediately frozen, and stored at -80°C until analysis.

A sandwich enzyme-linked immunosorbent assay (ELISA) kit (Biomatik Corporation, Cambridge, Ontario) was used to quantitatively assess CK8 in AH. Samples were added to a 96 well microplate containing biotin-conjugated antibody specific to CK8. Avidin conjugated to horseradish peroxidase was added to each well, and the microplate was incubated. The addition of TMB substrate solution then generated a color change only in wells containing CK8 biotin-conjugated antibody and enzyme-conjugated avidin. After terminating the reaction with sulfuric acid solution, the color change was measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. Sample CK8 concentration was then determined via comparing optical density of samples to the standard curve.

Imaging and data analysis

Neovascular AMD was diagnosed using FP, FA, ICGA and OCT, with hyperfluorescence and late leakage associated with detachment of pigmented epithelium, serous retinal detachment, subretinal exudation, and SMH. Choroidal neovascularization was subdivided into four categories. First, polypoidal choroidal vasculopathy (PCV) was diagnosed based on ICGA, specifically the presence of

a branched vascular network, along with terminal polypoidal lesions, sub-pigment epithelial layer orange-red protrusions corresponding to those polypoidal lesions, or both. Next, type 1 CNV was characterized by new vessels beneath the RPE, while type 2 CNV was defined as new vessels penetrating the RPE layer and localizing in the subretinal space on OCT. Finally, Type 3 CNV, or retinal angiomatous proliferation, was the intraretinal proliferation of new vessels originating from both retinal and choroidal circulation. The presence of retinal-choroidal anastomosis is identified with ICGA, intraretinal hemorrhages on FP, or intraretinal fluid on OCT. Boundaries of CNV was delineated using FA, allowing for the calculation of CNV area. Central subfield macular thickness (CMT) was automatically calculated with OCT as the average retinal thickness within the center circle of the Early Treatment Diabetic Retinopathy Study grid; this is a 1000 μm-diameter circle centered on the fovea. Choroidal thickness (ChT) was measured under the foveal center, vertically from the outer border of the RPE hyper-reflective line to the sclera inner border. Morphological features were also evaluated with FP or OCT, including presence of intraretinal fluid (IRF), subretinal fluid (SRF), SMH, drusen, and hard exudate.

Visual and anatomical treatment outcomes were evaluated with BCVA and OCT 3 months after initial injection (Month 3). The visual outcome was BCVA at Month 3 using the Snellen visual acuity chart, converted to logarithm of minimal angle of resolution units (logMAR). Anatomical outcome was OCT detection of fluid (IRF or SRF) presence at Month 3, with persistent fluid being a poor outcome. All measurements and diagnosis were conducted by retinal specialists (JYS and JL). Evaluations used average values.

Statistical analysis

Between-group differences in baseline characteristics and CK8 were determined with independent t tests (two-tailed) for continuous variables and Chi-squared tests for categorical variables. Pearson's correlations was used to evaluate the relationship between baseline CK8 and continuous baseline characteristics, while independent t tests were used to compare how baseline CK8 differed across

categorical baseline characteristics. A paired t-test compared CK8 before and after IVR, whereas another independent t-test compared CK8 between responders (dry nAMD eyes) and poor responders (persistent fluid). The association between CK8 and visual outcome was determined with linear regression, while the association between CK8 and anatomical outcome was determined with logistic regression. A multivariate regression was then performed, with the dependent variable being treatment outcome and independent variables being clinically significant parameters associated with treatment outcome in univariate analyses. All statistics were performed in SPSS for Windows (version 21.0; IBM Corp., Armonk, NY). Significance was set at $p < 0.05$.