

**TITLE PAGE**

**Optical Coherence Tomographic Characteristics of Microaneurysms in Diabetic Retinopathy**

**Short Title**

Microaneurysms in DR on OCT

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## TEXT

### INTRODUCTION

Diabetic retinopathy (DR) is a very serious and frequently occurring complication that causes severe visual loss in people of working age. Adequate prevention and therapy have not been established. Microaneurysms are hallmarks of the clinical diagnosis of DR, and the total number of microaneurysms indicates the likely progression to more severe retinopathy in the future,<sup>1-4</sup> which has led to the development of automated algorithms for their detection and calculation.<sup>5,6</sup>

Histopathologic studies, based on light microscopy or electron microscopy of sectional images and two-dimensional images of trypsin digestion preparation, have documented various changes in microaneurysms compared to the physiologic retinal vasculature; thick or thin basement membranes, loss of pericytes, and endothelial hypercellularity or acellularity.<sup>7-10</sup> Pathophysiologic changes, such as rheologic and fibrinolytic properties of the blood in patients with diabetes, also might affect microaneurysm formation and disappearance.<sup>11,12</sup> However, it is unclear how hyperglycemia induces these changes and the concomitant microaneurysm formation. Although microaneurysms are clinically known to be dynamic in nature,<sup>1,5,13</sup> it is unknown if the diversity in their appearance depends on their stages or origins.

Microaneurysms often present as hyperfluorescent dots on fluorescein angiography (FA) images, and some microaneurysms have been accompanied by focal leakage of fluorescence dye. Vascular hyperpermeability in DR may induce diabetic macular edema (DME) and reduced visual function, although the pathogenesis of the breakdown of inner blood-retinal barrier (BRB) and concomitant retinal edema remain to be elucidated. Clinically, focal photocoagulation of microaneurysms and grid photocoagulation applied to the diffuse leakage reduce the risk of poor visual prognosis,<sup>14-17</sup> suggesting that vascular leakage from microaneurysms contributes to the development or exacerbation of DME. However, not all microaneurysms are hyperpermeable, and we sometimes observe that microaneurysms with focal leakage are not accompanied by retinal edema. In addition, histopathological studies have not documented the detailed correlation between the characteristics of microaneurysms and retinal edema.

Optical coherence tomography (OCT) is a noninvasive technique for obtaining cross-sectional retinal images for the diagnosis and management of retinal diseases.<sup>18</sup> Several years after the introduction of time-domain OCT, spectral-domain OCT (SD-OCT), which provides images with an axial resolution of less than 5 microns, became available, albeit not widely.<sup>19</sup> SD-OCT, with a substantially faster rate of image acquisition and multiple B-scan averaging, may be useful for obtaining improved images of the retinal pathologic features, which might correspond to histologic sections

observed with light microscopy. SD-OCT identifies the individual retinal layers and provides much needed clinicopathological information.

In the current study, we characterized for the first time the *in vivo* images of microaneurysms using SD-OCT, investigated the relationship with nearby retinal cystoid spaces, and found a novel association between the vascular walls of the microaneurysms and the cystoid spaces.

## **METHODS**

### **Patients**

Consecutive 76 eyes of 60 patients (mean age,  $67.7 \pm 7.9$  standard deviation [SD] years; range, 50-83) who visited the Department of Ophthalmology of Kyoto University Hospital from September 2008 to March 2009 were evaluated retrospectively. Eyes with microaneurysms for which good quality fundus photographs, FA and OCT images were available were included. Twenty-two eyes presented with mild nonproliferative diabetic retinopathy (NPDR), 43 moderate NPDR, 9 severe NPDR, and two PDR. All research and measurements adhered to the tenets of the Declaration of Helsinki and were approved by the ethics committee of our institution. Informed consent was obtained from each patient after a detailed explanation of the nature and possible consequences of the study procedures.

### **Image Acquisition**

After a routine examination including fundus biomicroscopy and best-corrected visual acuity (BCVA) measurement, 45-degree (3,216 x 2,136 pixels) color fundus photographs were obtained using a fundus camera (TRC-50LX, Topcon, Tokyo, Japan), and the retinal sections were depicted using a SD-OCT, Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) with infrared fundus photograph. The foveal centered images of retinal sections were acquired with the 30 degrees crosshair mode or raster scan mode (20 degrees horizontal scan x 19 lines in vertical 20 degrees), and more than 20 scans were averaged using the manufacturer's software (Spectralis Acquisition and Viewing modules, version 4.0, Heidelberg Engineering). Thirty-degree FA images (1,536 x 1,536 pixels) were obtained using Heidelberg Retinal Angiography 2 (HRA2, Heidelberg Engineering).

The microaneurysms were seen as white, red, or multicolored dots in color fundus photograph, and one spot sometimes contained multiple colors, although the microaneurysms that had red spots can be confused with dot-like intraretinal hemorrhages.<sup>8,9</sup> The microaneurysms were detected as hyperfluorescent dots in the early or late phase compared with the fluorescence that is blocked by retinal hemorrhages.<sup>20</sup> However, it can be confusing that some microaneurysms detected in the color fundus photographs do not have hyperfluorescent dots and that conversely some hyperfluorescent dots are not visible in color photographs, and it is difficult to

differentiate these microaneurysms from dot-like hemorrhages.<sup>8-9,21</sup> We therefore investigated microaneurysms detected in both color fundus photograph and FA in this study, and found that all of them were well demarcated, round or oval lesions on the OCT images, whereas all the blot-like intraretinal hemorrhages had blurred margins, as reported in histopathological studies.<sup>22</sup> We also often observed cystoid spaces near the microaneurysms on the OCT images; cystoid spaces within 100  $\mu\text{m}$  of the edge of the microaneurysms were defined as being nearby ones. We further analyzed focal retinal edema; the localized retinal thickening with the area less than 2 disc area<sup>23</sup> which contained microaneurysm(s), confirmed by both biomicroscopy and OCT sectional images. We sometimes observed microaneurysms in diffuse edema, and they were excluded from this current study, because it was difficult to determine whether or not the microaneurysms contributed to these edematous changes.

### **Statistical Analysis**

The results are expressed as the mean  $\pm$  SD. Significant differences in sampling distribution were determined using the chi-square test. A *P* value of 0.05 was considered statistically significant.

## **RESULTS**

### **Microaneurysm Characterization**

We first evaluated microaneurysms, which were seen as hyperfluorescent dots on FA and which were inadvertently imaged on OCT, and found that all the intraretinal lesions were well demarcated and round or oval (Figs 1, 2), which agrees with clinicopathological reports that documented that microaneurysms appear saccular or fusiform with a circumscribed basement membrane.<sup>7-10</sup> The average values of horizontal and vertical diameter of microaneurysms on Spectralis OCT were  $118.3 \pm 43.3 \mu\text{m}$  and  $111.6 \pm 38.4 \mu\text{m}$  (ranged from 49.0 to 233.7  $\mu\text{m}$ , and 54.0 to 216.1  $\mu\text{m}$ , respectively) in this current study, which is, to some extent, compatible to the previous publications reporting their diameter (ranged from 14 to 200  $\mu\text{m}$ ).<sup>4-5,12,21,24,25</sup>

Although ophthalmologists are often confused by the similarity between microaneurysms with red spots and dot-like intraretinal hemorrhages on color fundus photographs, histologic reports have documented the differences between well-demarcated, round or oval microaneurysms and amorphous intraretinal hemorrhage with blurred margins.<sup>22</sup> Spectralis OCT also delineated such features as shown histopathologically, which seems useful for differentiating these lesions, in addition to FA (Fig 1, 2).

### **Capsular Structure of Microaneurysms**

Although the physiologic vascular walls are comprised of a basement membrane, a monolayer of endothelial cells, and pericytes, histopathology has shown the heterogeneity in these components, i.e., thin or thick basement membranes and

multilayers or no endothelial cells.<sup>7,10,22,25</sup> These reports prompted us to evaluate the status of the vascular wall of microaneurysms on Spectralis OCT sectional images. The capsular structure with a high signal intensity completely circumscribed the round or oval spaces in 28 microaneurysms (referred to as the complete ring sign on the sectional images of Spectralis OCT) (Fig 3). Compared to these microaneurysms, the ring sign was absent in 65 microaneurysms, and 54 microaneurysms were demarcated partially with the capsule (referred to as the incomplete ring sign) (Fig 3). Intriguingly, Spectralis OCT sometimes delineated the hyperreflective spots in the lumen of microaneurysms, which might be compatible to the previous reports demonstrating that their lumen was often filled with erythrocytes or leucocytes (Fig 3).<sup>25</sup> Furthermore, 41 (63.1%) of 65 microaneurysms with no ring sign had hyperreflective spots, whereas these spots were delineated only in 12 (42.9%) of 28 microaneurysms with complete ring sign and 22 (40.7%) of 54 microaneurysms with incomplete ring sign, which showed the significant differences in the frequency ( $p=0.033$ ; Table 1).

#### **Relationship between microaneurysms and retinal edema**

We then investigated the relationship between the status of the capsular structure of the microaneurysms and focal retinal edema, but could not find the significant association between them (Table 1). We further evaluated the association between their ring sign and the nearby cystoid spaces. Thirty-nine (60.0%) of 65 microaneurysms with no ring sign were accompanied by one or more cystoid spaces, whereas nearby cystoid spaces were observed in only 7 (25.0%) of 28 microaneurysms with a complete ring sign (Table 1), which showed the significant differences in the frequency. Interestingly, the signal intensity of these cystoid spaces was often heterogeneous (Fig 4), and microaneurysms with either an incomplete or no ring sign were accompanied by the cystoid spaces more frequently than those with a complete ring sign, but the difference was not significant (Table 1).

#### **Microaneurysms Adjacent to or Protruding into Cystoid Spaces**

When we investigated the relationship between microaneurysms and cystoid spaces, we found that 13 (17.3 %) of 75 microaneurysms accompanied by cystoid spaces protruded into the cystoid spaces, whereas another 62 microaneurysms were simply adjacent to them (Fig 4). Most microaneurysms that protruded into the cystoid spaces had no ring sign, and those that were adjacent to the cystoid spaces had a heterogeneity in their ring sign, suggesting an interaction between the capsular structure of microaneurysms and the cystoid spaces. However, we could not find a significant difference in the heterogeneity of the signal intensity in the nearby cystoid spaces between the two types (Table 2).

#### **Microaneurysms in the Retinal Layers**

The vasculature in the human retina is in the ganglion cell layer (GCL) and the inner and outer borders of the inner nuclear layer (INL),<sup>26</sup> and immunohistologic analysis found

that microaneurysms in DR originated predominantly from the INL.<sup>24</sup> We also assessed in which layer the center of the microaneurysms was located and found that they resided mainly in the INL (80.3%), and some (0.7%) were in the nerve fiber layer (NFL), the GCL/inner plexiform layer (15.0%), or the outer plexiform layer (OPL)/outer nuclear layer (4.1%), and the ring sign was absent in 54 (45.8%) of 118 microaneurysms in INL and 11 (37.9%) of 29 microaneurysms in other layers (Fig 5, Table 3). Since the signal intensity of the microaneurysms was similar to that of the NFL, it was difficult to detect those in the NFL, and their distribution might not be correct. Since histopathological reports have documented that cystoid spaces emerge predominantly from the INL and OPL,<sup>27</sup> we investigated the association between the location of the microaneurysms and the cystoid spaces. Nearby cystoid spaces were observed in 65 microaneurysms (55.1%) in INL and 10 microaneurysms (34.5%) in other layers.

## DISCUSSION

In the current study, we showed for the first time the characteristics of the microaneurysms in OCT images and the association of the microaneurysms with the nearby cystoid spaces. Despite their relevance to the diagnosis of DR, the definition of the microaneurysms is confusing, because not all microaneurysms detected by fundus microscopy or color fundus photography have hyperfluorescent spots and vice versa.<sup>8-9,21</sup> This discrepancy depends on several factors. Microaneurysms sometimes have a color similar to the fundus, which prevents differentiation from the fundus background. Since some microaneurysms are obstructed or nonperfused, fluorescent dye cannot fill the lumens, resulting in no hyperfluorescent dots. In the current study, we investigated the characteristics of the microaneurysms on Spectralis OCT corresponding to those detected by color fundus photography. All microaneurysms were distinguished easily by their specific characteristics in size, shape, and margin compared to other intraretinal lesions in DR. Since we evaluated the microaneurysms detected only by crosshair or raster scan mode, we did not delineate all characteristics of microaneurysms, and could not determine whether the images were sectioning through the center of microaneurysms, even if we tried the highest scan density using Spectralis OCT. Although it might be the limitation of this generation of OCT, improvements in OCT technology might enable us to obtain continuous three-dimensional scans of all retinal areas in the future. If so, high-resolution OCT would delineate all microaneurysms and provide the most useful information at the diagnosis of DR, in addition to fundus biomicroscopy and FA.

We next classified microaneurysms into three types according to the status of the capsular structure, the ring sign. The ring signs had varying thicknesses and reflectivities, which might correspond to the characteristics of their components (vascular walls, basement membrane, endothelial cells, or pericytes). Histopathological

reports have documented a number of characteristics of basement membranes (thick or thin, hyalinized, fibrous, laminated or lipid-containing), and the status of endothelial cells (hypercellular or acellular and multilayered or single layered).<sup>7-10,24-25</sup> A thick ring sign might correspond to a thickened and hyalinized, fibrous or lipid-containing basement membrane or multilayered endothelial cells. The high intensity of the ring sign might reflect the constituents of the basement membrane or intramural hard exudates or specific cellular components as reported previously.<sup>28</sup> However, we did not evaluate these features, because these parameters were often modified by the averaging protocol in Spectralis OCT and we cannot prove that the path of the OCT sectional images passes through the center of the microaneurysms. Further clinicopathological studies are needed to elucidate that relationship.

We further observed the hyperreflective spots in the lumens of the microaneurysms, which were abundant in the microaneurysms with no ring sign (Fig 3). Pathological studies have shown that the cellular components in the lumens of the microaneurysms are erythrocytes or polymorphonuclear leukocytes and intramural cells, endothelial cells, or pericytes<sup>8-9,25</sup> and that dense hyalinized microaneurysms are often acellular, whereas hypercellular microaneurysms have a thin wall.<sup>8</sup> These hyperreflective spots might correspond to these cells, although we could not determine the cell types exactly. Microaneurysms with no ring sign contained these spots more often than those with either a complete or incomplete ring sign. This association between vascular wall and cellular contents in microaneurysms suggests that there are multiple types of microaneurysms *in vivo*, as reported.<sup>7-10,24,25</sup> They could originate differently or be the same lesions in different stages, which would be elucidated by longitudinal OCT observation.

Microaneurysms with a complete ring sign were accompanied by nearby cystoid spaces less frequently. If the capsular structure was comprised mainly of a thickened and hyalinized basement membrane, the microaneurysms often might be obstructed or nonperfused with resultant less permeability.<sup>7-10</sup> Another possible explanation could be that thick vascular walls represent the endothelial cells, which provide a barrier between the intravascular and extravascular spaces.<sup>29</sup> These speculations could be clarified by a clinical comparison between FA and OCT images or clinicopathological studies using high-resolution OCT. We also observed that microaneurysms with an incomplete or no ring sign often were accompanied by cystoid spaces with a heterogeneous signal intensity, suggesting that multiple patterns of BRB breakdown are involved in these microaneurysms. The hyperpermeability in the retinal vasculature depends on the increased endothelial transcellular transports or paracellular flux in the vascular endothelium.<sup>29</sup> In addition to these mechanisms, the direct flow of blood constituents might happen through ruptured microaneurysms,<sup>10</sup> because we have observed that the

microaneurysms can be connected to a more reflective mass in the heterogeneous cystoid spaces (Fig 4).

Among 75 microaneurysms accompanied by cystoid spaces, 13 microaneurysms protruded into the cystoid spaces, and 11 of 13 (84.6%) (Table 2) did not have a capsular structure. It is reasonable that hyperpermeability of the microaneurysms with no ring sign developed or exacerbated the cystoid spaces. However, the capsular structure differed significantly between the microaneurysms that protruded into the cystoid spaces and those that were adjacent to the cystoid spaces, although both were associated with the cystoid spaces. Conversely, the effects of the cystoid spaces on the microaneurysms should be considered; the absence of a surrounding retinal parenchyma might lead to weak points in the basement membrane and loss of the survival factors of the endothelial cells. The factors that determine the characteristics of such microaneurysms need to be investigated further.

We could not find the significant association between the ring sign of microaneurysms and focal retinal edema, compared to that with nearby cystoid spaces. We speculated that focal edema often contains several microaneurysms, which might abolish the association between them. Retinal thickening might depend on the several factors; vascular permeability, retinal ischemia, structural fragility, the accumulation of extracellular fluids, or cytoplasmic swelling of Müller cells. Direct coagulation to the microaneurysms with each type of ring sign would elucidate the contribution to focal edema.

In the current study, we classified the microaneurysms according to appearance and found a novel association with nearby cystoid spaces. Interestingly, it appears that some microaneurysms are ruptured and that the blood constituents flow directly into the cystoid spaces, which is a novel pathophysiologic mechanism of the breakdown of the inner BRB. These data would shed light on the pathogenesis of DME, with resultant improvements in focal photocoagulation, the conventional treatment.

## **ACKNOWLEDGMENT**

a. Funding/Support: none.

b. Financial Disclosures: none

c. Contributions to Authors: Design of the study (TM, KN, AS, NY); Conduct of the study (TM, KN, AS, MO, TH, NY); Collection of data (TM, KN, AS, MO, TH, NY); Interpretation of data (TM, KN, AS, NY); statistical analysis of data (TM, KN, AS, NY); and preparation, review or approval of the manuscript (TM, KN, AS, MO, TH, NY).

d. Statement about Conformity with Author: All the research and measurements were adhered to the tenets of the Declaration of Helsinki, and approved by the Institutional Review Board at Kyoto University Graduate School of Medicine. The informed consent was obtained from each patient after the detailed explanation of the nature and possible consequences of the study procedures.

e. Other Acknowledgment: none.

**APPENDIX**

None

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## FIGURE CAPTIONS

### **Figure 1. Microaneurysms in diabetic retinopathy.**

(Top left) An infrared image of a microaneurysm. (Top right) The microaneurysm is oval, well-demarcated lesion (red arrow) compared to the intraretinal hemorrhages (red arrowhead) in the optical coherence tomography image corresponding to the green line in the infrared image. A magnified image of a microaneurysm (Middle center) and an intraretinal hemorrhage (Middle right). The blue arrowheads: the cystoid spaces. (Middle left) A microaneurysm in a color fundus photograph and its magnified image (bottom left; black arrow). Early-phase (Bottom center) and late-phase (Bottom right) fluorescein angiography. The black and red arrows indicate microaneurysm.

### **Figure 2. A microaneurysm with capsular structure in diabetic retinopathy.**

(Top right) The microaneurysm is circumscribed by a capsular structure (arrow) in the optical coherence tomography image corresponding to the green line in the infrared image in Top left, and the magnified image of the microaneurysm (Middle right). (Middle left) A microaneurysm in a color fundus photograph in the outlined area and its magnified image (Bottom left). Early-phase (Bottom center) and late-phase (Bottom right) fluorescein angiography. The black and red arrows indicate microaneurysm.

### **Figure 3. Three types of capsular structures (ring sign) in a microaneurysm in diabetic retinopathy.**

(Top) A microaneurysm is continuously circumscribed by a high-intensity capsular structure (complete ring sign). The arrow indicates the microaneurysm. (Middle) The capsular structure is not continuous (arrowhead) around the microaneurysm (incomplete ring sign). The arrow indicates the microaneurysm. (Bottom) The capsular structure is not delineated at all (absent ring sign). Hyperreflective spots are in the microaneurysm. The arrow indicates the microaneurysm.

### **Figure 4. Microaneurysms adjacent to or protruding into the cystoid spaces in diabetic retinopathy.**

(Top row) The microaneurysm (white arrow) is adjacent to the cystoid spaces. (Bottom row) The microaneurysm (white arrow) protrudes into the cystic spaces. The black arrowheads indicate cystoid spaces. (Right column) The cystoid spaces have a heterogeneous signal intensity (black arrows), and the microaneurysm is sometimes connected to the high-intensity area in the cystoid spaces. The white arrow indicates the microaneurysm.

### **Figure 5. A microaneurysm in the retinal layer in diabetic retinopathy.**

(Top) A microaneurysm in the nerve fiber layer (NFL); (Middle top) ganglion cell layer/inner plexiform layer (GCL/IPL); (Middle bottom) inner nuclear layer (INL); (Bottom) outer plexiform layer/outer nuclear layer (OPL/ONL). The arrow indicates a microaneurysm. RPE, retinal pigment epithelium.