The nature of the white opaque substance within colorectal neoplastic epithelium as visualized by magnifying endoscopy with narrow-band imaging

Background and study aims: We previously reported our discovery of a white opaque substance (WOS) that is opaque to endoscopic light inside the epithelium while using magnifying endoscopy (ME) to examine gastric epithelial neoplasia. Histopathologic analysis revealed that the WOS comprised minute lipid droplets (LDs) accumulated within the neoplastic epithelium. In addition, the WOS was found in colorectal epithelial neoplasia, although it was unclear whether this WOS corresponded to an accumulation of LDs, as in the stomach. Therefore, the aim of the current study was to elucidate whether the WOS observed in colorectal epithelial tumors comprises LDs.

Patients and methods: A consecutive series of 40 WOS-positive and 40 WOS-negative colorectal epithelial tumors was analyzed. One biopsy specimen was taken from each neoplasm. Cryostat sections were stained with oil red O for LD, and sections after formalin-fixation for LD were immunostained with anti-adipophilin antibody.

Results: The prevalence of LDs stained with oil red O in WOS-positive vs. WOS-negative lesions was 47.5% (19/40) vs. 5% (2/40), respectively ($P<0.001$). Furthermore, the WOS coincided with the expression of adipophilin; the prevalence of LDs stained by anti-adipophilin antibody in WOS-positive vs. WOS-negative lesions was 100% (40/40) vs. 62.5% (25/40), respectively ($P<0.001$).

Conclusions: This study elucidated for the first time that endoscopically visualized WOS in colorectal epithelial neoplasia may be composed of LDs accumulated in the neoplastic epithelium.
The exclusion criteria were: (1) neoplastic lesions with erosion, ulceration, or bleeding that disturbed the endoscopic observation of surface morphology; (2) serious underlying disorders; (3) a past history of colectomy; (4) receipt of anti-thrombotic agents and high risk of bleeding from forceps biopsy; and (5) ineligible to participate in the study as determined by the investigators. A total of 240 patients with colorectal epithelial tumors (adenomas and carcinomas) underwent EMR and ESD at the Department of Endoscopy of the Fukuoka University Chikushi Hospital between July 2013 and February 2014. The study gathered data on a target population of 40 consecutive WOS-positive and 40 consecutive WOS-negative patients who met the above criteria.

Endoscopic procedures and biopsy

All endoscopic procedures were performed using an electronic endoscopy system (Evis-Lucera Spectrum; Olympus Medical Systems Co., Tokyo, Japan) with magnifying colonoscopy (CFH260AZI, PCF-Q240ZI; Olympus). A soft black hood attachment was mounted at the tip of the scope. The lesion was visualized using incremental movements of the tip of the endoscope to bring the image into focus, with a distally attached soft black hood to stabilize the tip of the endoscope to bring the image into focus, with a distally attached soft black hood to stabilize the lesion. All patients were given 2 liters to 3 liters of polyethylene glycol-electrolyte solution on the morning of the examination day as preparation. When a colorectal epithelial lesion was detected during non-magnifying observation with white light imaging, the tumor surface was washed and immediately examined by ME with NBI to determine whether a WOS was present in the colorectal epithelial tumor. A WOS was defined as a white substance within the colorectal superficial epithelium that obscured the subepithelial microvascular pattern. The presence or absence of a WOS was investigated under maximal magnification (Fig. 1 and Fig. 2). When a WOS was identified in the surface layers of the most anal part of the colorectal epithelial tumor, the lesion was assessed as WOS-positive, and targeted biopsies were subsequently taken from this part of the tumor. Macroscopic classification was done according to the updated Paris classification [8]. All biopsy specimens were embedded in Tissue-Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and immediately frozen in acetone (SigmaAldrich, CAS. No. 67-64-1, St. Louis, USA) stored at −60 °C. Serial frozen sections (5-μm-thick) were cut with a cryostat and mounted on commercially available, charged adhesive coating slides (PRO-12, Matsunami Glass Ind., Ltd, Osaka, Japan) for oil red O staining. The remainder of each frozen specimen was fixed with 20% formalin overnight and embedded in paraffin. Some serial sections (5-μm thick) were cut with a microtome. One was stained with hematoxylin and eosin (HE) for standard histological investigations, and the others were prepared for immunohistochemistry.

Oil red O staining

To investigate lipid accumulation in the colorectal neoplasms, LDs were observed using oil red O staining performed as described previously [9] with some modifications.

Immunohistochemical staining

For immunostaining, an automatic and clinically validated instrument based on the Ventana Benchmark ULTRA system (Roche Tissue Diagnostics, Basel Switzerland) was used. Immunohistochemistry was performed with a new, enhanced-sensitivity, biotin-free multimer technology system, based on direct linkers between alkaline phosphatase and secondary antibodies (ultraView Universal Alkaline Phosphatase Red Detection Kit, Ventana Medical Systems, Basel Switzerland). In addition to oil red O staining, to assess lipid accumulation in the colorectal neoplasms, the primary antibody against adipophilin (1:50, AP125, Acris Antibodies, San Diego, CA, USA) was used. To classify phenotypic expression, the following primary antibodies were used: CD10 (1:20, 56C6, Leica Biosystems, Newcastle, UK), MUC2 (1:100, Ccp58, Leica Biosystems), CDX2 (1:50, CDX2-88, BIOCARE...
MEDICAL, Concord, CA, USA), MUC5AC (1:100, CLH2, Leica Biosystems), and MUC6 (1:100, CLH5, Leica Biosystems). Expression was classified as positive (negative) when more (fewer) than 5% of the neoplastic cells in the neoplastic areas were stained.

**Immunoelectron microscopy**

Immunoelectron microscopy was carried out on WOS-positive colonic neoplasms based on a modified Yano-Kashima method (pre- and post-embedding method) [10]. From the representative paraffin-embedded blocks of tumor specimens, sections were cut at 3-μm thickness and mounted on silane-coated glass slides. All sections were deparaffinized in xylene and rehydrated in a graded ethanol series. After blocking of nonspecific reactivity with protein block solution (Protein Block Serum-Free; DAKO, Tokyo, Japan) for 7 min at room temperature (RT), sections were incubated overnight at 4°C with the anti-adipophilin monoclonal antibody (1:10; clone AP125, Acris Antibodies GmbH, Hidden- och, Germany). Distribution of the primary antibody was achieved with subsequent application of a biotinylated anti-primary antibody (Histofine SAB PO kit; Nichirei, Tokyo, Japan). These sections were fixed in 2% glutaraldehyde for 1 h at RT and postfixed in 1% OsO4 for 0.5 h at RT. After being dehydrated in an ascending graded series of ethanol, the sections were embedded in epoxy resin (Epok812; Okenshoji, Tokyo, Japan) with DMP-30 (TAAB Laboratories; Berkshire, UK), which was polymerized by heating at 40°C for 12 h and then at 60°C for 24 h. Ultrathin sections were cut with an ultramicrotome (Leica Instruments; Nuss-loch, Germany) at a thickness of 90 nm and mounted on nickel grids. Antigen retrieval using Target Retrieval Solution (pH 10, DAKO) was carried out according to the previous Yano-Kashima method. After incubation with the anti-biotin monoclonal antibody (1:100; clone BK-1/39, DAKO) for 2 h at RT, the sections were incubated with 15-nm gold particle-conjugated anti-mouse IgG goat antibody (1:10, Amersham, Little Chalfont, Buckingham-shire, UK) for 0.5 h at RT. The immunostained ultrathin sections were counterstained with uranyl acetate and lead citrate as usual, and then examined using a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan).

**Histopathologic analysis**

Standard histologic diagnosis and histologic investigation of LDs and adipophilin, as well as the phenotypic classification of neoplasia were performed by a single pathologist (K. I.) who was blinded to the endoscopic findings. Diagnosis was based on either biopsied specimens or resected specimens according to the revised Vienna classification [11]. Presence or absence of LDs was determined by histologic analysis of the section stained with oil red O and immunostained with anti-adipophilin antibody. Neoplastic phenotype was classified as: intestinal (I) type if the neoplastic epithelium was positive for either CD10, MUC2, or CDX2; gastric (G) type if it was positive for either HGM, MUC5AC, or MUC6; or gastrointestinal (GI) type if it showed both gastric and intestinal phenotypes [12, 13]. Immunoelectron microscopic findings were investigated by a single pathologist (K. I.) who was blinded to the endoscopic findings.

**Interobserver and intra-observer agreement**

Forty WOS-positive (WOS+) and 40 WOS-negative (WOS–) neoplasms were extracted from among the subjects for use in the testing of interobserver agreement. The images were arranged in random order. First, to determine interobserver agreement, 2 independent endoscopists with 22 years (T. N.) and 12 years (T. K.) of experience in gastric endoscopy were asked to review all of the images and to determine whether each image represented the presence or absence of WOS [14]. Then, to determine intra-observer agreement, the first endoscopist was asked to review all of the images and to determine whether each image represented the presence or absence of WOS [14].

**Statistical Analysis**

Mean and median values were compared using the Student’s t-test and the Mann–Whitney U test, respectively. Comparisons of the prevalence between two groups were made using the Chi-squared test or Fisher’s exact test. P values < 0.05 were considered significant. SPSS version 21 J for Windows (SPSS, Chicago, IL, USA) was used for all statistical analyses.

**Results**

To gather data on the 40 consecutive WOS-positive patients (WOS-positive [WOS+] and 40 consecutive WOS-negative patients (WOS-negative [WOS–]), the 240 patients who underwent treatment between July 2013 and February 2014 were identified. From the 187 WOS– patients, the first 40 patients were included, and the latter 147 patients were then excluded (Fig. 3).
Table 1 Clinical features of colorectal neoplasia.

<table>
<thead>
<tr>
<th></th>
<th>WOS-positive (n = 40)</th>
<th>WOS-negative (n = 40)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>23/17</td>
<td>24/16</td>
<td>0.820</td>
</tr>
<tr>
<td>Age, mean ± SD (year)</td>
<td>69.3 (10.8)</td>
<td>63.8 (11.13)</td>
<td>0.027</td>
</tr>
<tr>
<td>Tumor size, mean ± SD (mm)</td>
<td>11.2 (5.51)</td>
<td>9.8 (5.5)</td>
<td>0.245</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal colon¹</td>
<td>24</td>
<td>11</td>
<td>0.003</td>
</tr>
<tr>
<td>Distal colon²</td>
<td>8</td>
<td>25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rectum</td>
<td>8</td>
<td>4</td>
<td>0.348</td>
</tr>
<tr>
<td>Macroscopic classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-Ip, 0-Ia</td>
<td>29</td>
<td>39</td>
<td>0.003</td>
</tr>
<tr>
<td>0-Ia, IIC, IIA + IIC</td>
<td>11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoma</td>
<td>32</td>
<td>37</td>
<td>0.192</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>8</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

WOS, white opaque substance; SD, standard deviation; 0-Ip, superficial protruding pedunculated type; 0-Ia, superficial sessile type; 0-Ia, superficial slightly elevated type; 0-IIC, superficial slightly depressed type; 0-IIa + IIC, superficial elevated and depressed type.

¹ Proximal colon, including cecum, ascending colon, and transverse colon.

² Distal colon, including descending colon and sigmoid colon.

Table 2 Histologic prevalence of lipid droplets by oil red O staining according to the presence of WOS on ME with NBI.

<table>
<thead>
<tr>
<th>Lipid droplets</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>WOS Positive (n = 40)</td>
<td>19 (47.5%)</td>
<td>21 (52.5%)</td>
</tr>
<tr>
<td>WOS Negative (n = 40)</td>
<td>2 (5%)</td>
<td>38 (95%)</td>
</tr>
</tbody>
</table>

ME, magnifying endoscopy; NBI, narrow-band imaging; WOS, white opaque substance. P<0.001, Fisher’s exact test.

Table 3 Immunohistochemical expression of adipophilin according to the presence of WOS on ME with NBI.

<table>
<thead>
<tr>
<th>Adipophilin</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>WOS Positive (n = 40)</td>
<td>40 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>WOS Negative (n = 40)</td>
<td>25 (62.5%)</td>
<td>15 (37.5%)</td>
</tr>
</tbody>
</table>

ME, magnifying endoscopy; NBI, narrow-band imaging; WOS, white opaque substance. P<0.001, Fisher’s exact test.

Prevalence of LDs, as histologically visualized by oil red O staining, in WOS+ vs. WOS- lesions was 47.5% (19/40) and 5% (2/40), respectively (P<0.001) (Table 2, Fig. 4). All the 40 WOS+ neoplasms (100%) were positive for adipophilin, whereas 25 of 40 WOS- neoplasms (62.5%) were positive for adipophilin (P<0.001) (Table 3, Fig. 5a, Fig. 5b). Median adipophilin density (range) within the epithelium of the WOS+ group and WOS- group was 8.4% (1.1–34.6%) and 4.0% (0–15.1%), respectively (Fig. 6). Adipophilin density was therefore significantly higher in the WOS+ group than in the WOS- group (Fig. 6) (P<0.001).

In 2 WOS+ adenomas examined, immunoelectron microscopy for adipophilin revealed numerous vacuoles in the neoplastic cells, especially in the subnuclear cytoplasm (Fig. 7a, Fig. 7b, Fig. 7c, Fig. 7d). The size of most vacuoles varied from 0.2 μm to 2.3 μm. At high magnification, the morphology of the vacuoles was round and polygonal (Fig. 7c, Fig. 7d). Significant numbers of gold particles were observed in the inner sides of the vacuoles (Fig. 7c, Fig. 7d), showing a positive immunoreaction for adipophilin. In summary, the vacuoles represented LDs.

Assessment of mucin phenotypes indicated that the gastric type, gastrointestinal mixed type, and intestinal type occurred in 0 (0%), 8 (20%), and 32 (80%) WOS+ neoplasms, and in 0 (0%), 11 (27.5%), and 29 (72.5%) WOS- neoplasms, respectively. There was no association between the prevalence of WOS and a particular phenotype (Table 4).

The interobserver agreement/kappa value for assessment of WOS+ and WOS- neoplasms using ME with NBI was 0.85, indicating excellent agreement. The intra-observer agreement/kappa value was 0.92, also indicating excellent agreement.

Discussion

The current study demonstrated for the first time that endoscopically-visualized WOS on the surface of large intestinal epithelial neoplasms comprised LDs accumulated in the neoplastic epithelium. Oil-red O staining showed that LDs were present in 47.5% (19/40) of the tumor sites that tested positive for WOS (WOS+) and in only 5% (2/40) of the tumor sites that tested negative for WOS (WOS–). Prevalence of WOS and prevalence of LDs detected by fat staining were significantly correlated (P<0.001).
The hypothesis that WOS comprises LDs was also tested by immunostaining and immunoelectron microscopy with anti-adipophilin antibody, which can be used to identify the membrane protein of LDs [15, 16]. The immunostaining results showed that adipophilin was expressed in 100% (40/40) of WOS+ neoplasms and in 62.5% (25/40) of WOS− neoplasms, thus demonstrating a significant correlation between prevalence of WOS and prevalence of LDs detected by immunostaining with adipophilin (P < 0.001). However, given that adipophilin was also detected in 62.5% (25/40) of the WOS− group and WOS− group is 8.4% (1.1 - 34.6) and 4.0% (0 - 15.1), respectively. Adipophilin density is significantly greater in the WOS+ group than in the WOS− group (P < 0.001, Mann-Whitney U test).

Using immunoelectron microscopy, Ueo et al. found that the size of LDs accumulated in gastric epithelial neoplasms ranged from 0.1 μm to 4 μm [17]. Meanwhile, the size of LDs in large intestinal epithelial neoplasms measured with immunoelectron microscopy in the current study was 0.2 μm to 2.3 μm, implying that the LDs in large intestinal epithelial neoplasms tend to be smaller than those in gastric epithelial neoplasms. Mehlem et al. described the difficulty of detecting LDs with oil-red O staining when their accumulation is sparse [18]. We therefore considered that the detection rate of oil-red O-stained LDs in large intestine WOS+ neoplasms was lower than that of gastric neoplasms due to the low sensitivity of oil-red O staining of LDs in large intestinal epithelial neoplasms owing to the small size of the droplets themselves. The findings of the current study based on oil-red O staining, immunostaining with anti-adipophilin antibody, and immunoelectron microscopy suggest that the WOS in fact comprises accumulation of LDs within the neoplastic epithelium.

One limitation of the current study is that the chemical composition of these LDs remains unclear. Hong et al. reported that enhanced visualization of small peptides absorbed through a rat intestinal membrane was achieved by matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-IMS) with the aid of phytic acid as a matrix additive, and they established phytic-acid-aided MALDI-IMS for the visualization of small peptides absorbed through a rat intestinal membrane [19]. Application of these techniques would allow us to identify the chemical composition of the WOS accumulated in human large intestine epithelia in the future. We are planning to use these techniques in a separate study to determine the chemical composition of these LDs.

While the mechanism responsible for epithelial accumulation of LDs remains uncertain, there are currently 2 hypotheses [4]. One hypothesis, known as the “absorption theory,” attributes the accumulation to exogenous lipids absorbed by passive diffusion from the neoplastic epithelium, and the other hypothesis, known as the “synthesis theory,” attributes the mechanism to endogenous lipids, whereby the tumor cells themselves are responsible for synthesizing the LDs. Since orally absorbed lipids are digested and largely absorbed by the small intestine, their presence in the large intestine is minimal. However, intestinal bacteria are known to produce short-chain fatty acids in the large intestine.
as a metabolic product of dietary fiber. Ninety-five percent or more of short-chain fatty acids produced in the gastrointestinal tract are absorbed by passive diffusion and carrier-mediated transport [20]. The current study demonstrated that endoscopically visualized WOS comprised LDs accumulated in the neoplastic epithelium in the large intestine; thus, the mechanism responsible for this WOS formation could be attributed to accumulation of LDs resulting from absorption of short-chain fatty acids in large intestinal epithelia. Meanwhile, activation of the endogenous fatty acid synthesis pathway is also known to occur in cancer cells [21]. Further studies are needed to clarify the pathological mechanism of lipid accumulation in large intestinal epithelial neoplasms.

Using electron microscopy, Accioly et al. showed that LDs were significantly more prevalent in neoplastic large intestine lesions than in non-neoplastic large intestine lesions [22]. Nevertheless, according to the report by Hisabe et al., WOS is present with a high prevalence within the epithelium of hyperplastic polyps [23]. Therefore, presence of WOS alone cannot be an optical marker for making a differential diagnosis between non-neoplasia and neoplasia.

With regard to neoplasia, a study by Hisabe et al. comparing WOS detection rates in adenoma and carcinoma showed a significantly higher rate of WOS-positive neoplasms in carcinoma (31.8% vs. 66.0%, respectively, P < 0.001). The study also compared WOS detection rates between intramucosal and submucosal carcinoma, and found that WOS prevalence was significantly higher in submucosal carcinoma (59.0% vs. 75.9%, respectively, P = 0.038) [5]. In addition, the morphology of WOS seemed to differ depending on the level of malignancy in the neoplasia, that is, WOS in adenomas, high-grade dysplasias and carcinomas with slight invasion of the submucosa had regular morphology, whereas irregular morphology was observed in carcinomas massively invading the submucosa [6]. These findings suggest that the morphology of the WOS in colorectal neoplasia might be a useful new optical marker of neoplastic malignancy, as well as in gastric neoplasia [2]. We have already begun a prospective feasibility study (UMIN000021167) to investigate whether the morphology of WOS can be used as an optical marker for differential diagnosis between adenoma and high-grade dysplasia/carcinoma.

**Conclusion**

In conclusion, the current study demonstrated for the first time that WOS visualized within the superficial part of large intestinal epithelial neoplasms using ME comprises LDs accumulated in the neoplastic epithelium.

**Competing interests:** None

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