Extremely Well-Differentiated Adenocarcinoma of the Stomach
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Extremely well-differentiated adenocarcinoma of the stomach: diagnostic pitfalls in endoscopic biopsy

Jongwon Lee, In-Seob Lee, Ji Yong Ahn, Young Soo Park, Jihun Kim
Departments of Pathology, Surgery, and Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

Background: Extremely well-differentiated adenocarcinoma (EWDA) is a deceptively bland-looking adenocarcinoma of the stomach. It often causes diagnostic problems, especially in endoscopic biopsy samples. To better recognize this deceptively bland lesion, we carefully reviewed a series of EWDA treated at our institution. Methods: A total of 55 specimens from 19 patients were obtained. Endoscopic, gross and microscopic features defining EWDA were described and documented. For comparison, hyperplastic polyp specimens were randomly selected and analyzed. Results: Most cases (18 of 19, 94.7%) were advanced gastric cancer (AGC) and primarily located in the body of the stomach (15 of 19, 79.0%). The majority of AGCs were non-ulcerated (11 of 18, 61.1%) with an undermining growth pattern and a relatively small mucosal involvement. Specific histologic features included an irregular glandular shape, an undulating apical cytoplasmic border, disproportionately large glands, a variably distended mucinous cytoplasm. Classical features, such as small infiltrating glands or desmoplastic reactions, were rarely observed. Identification of irregularly spaced nuclei and disruption of the foveolar epithelial structure, along with atypical features described above were helpful in making a diagnosis especially in gastric forceps biopsies. Conclusions: Awareness of the histomorphologic characteristics described in this report would lead to timely diagnosis and prevent repeated endoscopic procedures.

Key Words: Stomach neoplasms; Adenocarcinoma; Missed diagnosis

Materials and Methods

Case selection

EWDA was defined as neoplastic glands comprised of highly differentiated cells mimicking intestinal metaplasia or normal gastric foveolar epithelium with mild nuclear atypia, according to the definition by Yao et al. [1], applied with slight modification. EWDA cases were identified and collected through routine clinical practice. In the screening process, the intestinal-type EWDA cases presented as early gastric cancers (EGCs) with the well-known crawling-type or shaking-hand-type morphologies were excluded. We focused more on the identification of advanced EWDA cases that were relatively diagnostically urgent. The malig-
nant nature was confirmed either by surgically resected specimens or by unequivocal clinical presentations as metastatic disease. The confirming procedure was warranted because the histologic appearances of EWDA were not easily recognizable due to deceptively bland morphologies. After the confirmation of malignant nature, we collected all related pre-operative biopsy samples. Fourteen cases from the authors’ institutional archive from 2018 to 2021 were collected by two pathologists (JL and JK).

To estimate the prevalence of EWDA, we reviewed 608 consecutive surgically resected advanced gastric cancers (AGCs) treated at our institution in 2010. AGCs were selected because most of the archived cases were AGCs. Five of them (5 of 608, 0.08%) fulfilled the criteria for EWDA, thus total of 19 cases was available for our study design. Patient characteristics, surgical and endoscopic findings with follow-up data were obtained from the medical records of Asan Medical Hospital.

Endoscopic assessment

Endoscopic data from available cases was collected and reviewed by expert gastroenterology specialist (JYA). The tumors were then classified as subepithelial-tumor-like lesions, slightly elevated or depressed lesions, or AGC Borrmann types according to the widely accepted endoscopic definition.

Histologic assessment and statistical analysis

Hematoxylin and eosin-stained sections for pretreatment biopsies and surgical specimens were available for all, and two pathologists (JL and JK) independently evaluated their gross findings, histomorphology, pathologic TN stage, and lymphovascular invasion statuses. Any discrepancy was resolved in consensus sessions under a multiheaded microscope. The ulcer proportion, defined as area of gross ulceration divided by the area of the entire tumor, were calculated in surgically resected ulcerated EWDA and control group AGCs from the year 2010. We used a non-parametric test (Mann-Whitney) to determine the p-value for differences between the groups. Statistical analysis was performed using SPSS software ver. 18.0 (SPSS Inc., Chicago, IL, USA), with p < 0.05 considered statistically significant.

All cases were evaluated and documented for the following histologic features observed in EWDA (Fig. 1, Supplementary Fig. S1): inharmonious disproportionate glands, irregularly shaped glands, undulating apical mucin border, and markedly distended mucinous cytoplasm. Inharmonious disproportionate glands referred to glands disproportionately larger than surrounding non-neoplastic glands. Irregular glandular shapes indicated irregular glandular structures that were cut off or distorted. Underlying mucinous cytoplasm. Inharmonious disproportionate glands referred to glands disproportionately larger than surrounding non-neoplastic glands. Irregular glandular shapes indicated irregular glandular structures that were cut off or distorted. Undulating mucin border described irregular, wobbly border of apical mucin caps. Markedly distended mucinous cytoplasm of EWDA cells indicated very large tumor cells sometimes exceeding 40 times the size of mature lymphocytes.

Background mucosa was also microscopically studied in terms of the presence of atrophic gastritis or intestinal metaplasia in adjacent mucosa. The time intervals between the initial biopsy and treatment, type of treatment, and the number of procedures performed were recorded. The original diagnoses of pretreatment biopsies were collected and compared with the final diagnoses.

Immunohistochemical analysis

Formalin-fixed paraffin-embedded tissue blocks were available for all. To determine the tumor immunophenotype, immunohistochemical staining was performed using antibodies against MUC-5AC (1:100, mouse monoclonal, clone MRQ-19, catalog No. 292M-96, Cell Marque, Rocklin, CA, USA), MUC-2 (1:50, mouse monoclonal, clone Ccp58, catalog No. NCL-MUC-2, Novocastra, Newcastle upon Tyne, UK), MUC-6 (1:200, mouse monoclonal, clone CHL5, catalog No. NCL-MUC-6, Novocastra), CDX-2 (1:500, mouse monoclonal, clone EPR2764Y, catalog No. 235R-16, Cell Marque), c-erbB2 (1:8, mouse monoclonal, clone 4B5, catalog No. 790-4493, Ventana, Tusan, AZ, USA), p53 (1:1,000, clone DO-7, Dako, Glostrup, Denmark), Ki-67 (1:200, mouse monoclonal, clone MIB1, catalog No. M7240, Dako) and PTEN (1:100, rabbit monoclonal, clone 138G6, catalog No. 9559, Cell Signaling, Danvers, MA, USA). Expression of mucin core proteins and CDX-2 were investigated to help classification of tumors into gastric- or intestinal- subtypes. C-erbB2 immunohistochemistry was done to find out the potential therapeutic targets. We did p53 and Ki-67 immunohistochemistry to determine their helpfulness in identifying the EWDA. Finally, PTEN immunohistochemistry was performed to confirm the PTEN protein expression loss in the case (case No. 3) with a certain PTEN mutation. All staining procedures were performed using a Ventana autostainer according to the manufacturer’s instructions.

Cytoplasmic staining for mucin core proteins (MUC5AC, MUC2, and MUC6) and nuclear staining for CDX-2 were considered positive. C-erbB2 staining was evaluated based on traditional human epidermal growth factor receptor 2 (HER2) immunohistochemistry scoring guidelines [3]. Cases scored equivocal for C-erbB2 were tested for HER2 gene copy-number by silver-enhanced in situ hybridization. For scoring, we followed general guidelines for HER2 copy-number evaluation as described by Jeong et al. [4]. Two pathologists (JL and JK) independently scored.
Extremely well differentiated carcinoma • 65

the immunostaining, and any discrepancy was resolved by consensus. Selective next-generation sequencing (NGS) data were available for three cases (case Nos. 2, 3, and 9). NGS was performed according to our routine clinical targeted cancer panel as described previously [5].

RESULTS

Clinicopathologic features

The clinical information of the 19 cases were listed in Table 1.

The median age of the patients was 63 years (range, 31 to 81 years) and the male to female ratio was 5.3:1 (16:3). The majority of the tumors (15 of 19, 79.0%) were located in the body of stomach in contrast to the usual type gastric carcinomas in which antral location is more common. The median tumor size in the resected cases was 4.0 cm in the greatest dimension (range, 2.2 to 10.0 cm). Most of the cases were clinically or pathologically proven AGCs (18 of 19, 94.7%).

Most patients were treated with surgery and adjuvant chemotherapy (11 of 19, 57.9%). Surgery alone was performed in two

Fig. 1. Representative photomicrographs of extremely well-differentiated adenocarcinoma (EWDA). (A) Intestinal-type EWDA featuring goblet cells is observed in case No. 12. (B) Irregularly shaped glands (arrows) opposed to normal foveolar glands (arrowheads) are observed in case No. 4. (C) Inharmoniously large glands (arrows) are noticeable against benign foveolar glands (arrowheads) in case No. 4. (D) A focal mucosal opening giving way to larger, deeply seated glands is noted, resembling lobular endocervical glandular hyperplasia of the uterine cervix (case No. 17). (E) Extremely large neoplastic glands, at least 20 times the size of normal lymphocytes, are noted in case No. 2. An undulating apical mucinous border is also observed (arrows). (F, G) Case No. 3 shows bland-looking gastric-type EWDA glands (arrows) both in gastric and omental biopsy specimens (G). (H) Case No. 11 shows cystically dilated thin neoplastic glands invading the muscularis propria with gastritis cystica profunda-like portions in the submucosa. (I) Glandular cancerization (arrows) in the background of normal foveolar glands (arrowheads) is more commonly found in EWDA than in conventional gastric carcinomas.
patients (2 of 19, 10.5%). Four of the five initially metastatic cases received chemotherapy without gastric resection (4 of 19, 21.1%). Endoscopic submucosal dissection was performed on the EGC (case 1, 1 of 19, 5.3%). The majority of the surgically resected AGC cases infiltrated to the subserosa (pT3, 7 of 14, 50.0%). The other cases penetrated to the serosa (pT4a, 4 of 14, 28.6%) or invaded muscularis propria (pT2, 3 of 14, 21.4%). The EGC case invaded the submucosa (pT1b). Lymphovascular invasion was present in some resected specimens including the EGC case (5 of 15, 33.3%). Nodal metastasis was histologically identified in some of the surgically resected cases (5 of 15, 33.3%). Only one (case No. 14) of such cases showed a very minor (< 1%) component of moderately differentiated adenocarcinoma. Even in the metastatic lymph nodes, the tumors retained their extremely well-differentiated morphology.

Patients were followed up for variable time intervals, ranging from 0.5 to 121 (median, 19 months) (Table 1). Some patients were alive without evidence of disease at last contact (8 of 19, 42.1%), while others were lost to follow-up (5 of 19, 26.3%). Of the five patients with distant metastasis at the time of diagnosis, the majority died of the disease at 5 months, 12 months, and 15 months after initial chemotherapy (3 of 19, 15.7%). The others were alive with disease at last contact (3 of 19, 15.7%).

Table 1. Clinical information of 19 cases of EWDA

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Treatment</th>
<th>Follow-up (mo)</th>
<th>Status</th>
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<tbody>
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<td>F</td>
<td>ESD</td>
<td>17</td>
<td>NED</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>F</td>
<td>Chemotherapy</td>
<td>19</td>
<td>AWD</td>
</tr>
<tr>
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<td>31</td>
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<td>Chemotherapy</td>
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<td>DOD</td>
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<td>56</td>
<td>F</td>
<td>Surgery</td>
<td>35</td>
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<tr>
<td>5</td>
<td>60</td>
<td>M</td>
<td>Surgery</td>
<td>28</td>
<td>NED</td>
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<tr>
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<td>62</td>
<td>M</td>
<td>Surgery</td>
<td>23</td>
<td>NED</td>
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<tr>
<td>7</td>
<td>39</td>
<td>M</td>
<td>Surgery</td>
<td>26</td>
<td>NED</td>
</tr>
<tr>
<td>8</td>
<td>67</td>
<td>M</td>
<td>Chemotherapy</td>
<td>14</td>
<td>DOD</td>
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<tr>
<td>9</td>
<td>72</td>
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<td>AWD</td>
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<td>69</td>
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<td>16</td>
<td>NED</td>
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<tr>
<td>11</td>
<td>58</td>
<td>M</td>
<td>Surgery</td>
<td>8</td>
<td>NED</td>
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<tr>
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<td>61</td>
<td>M</td>
<td>Surgery</td>
<td>15</td>
<td>NED</td>
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<tr>
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<td>48</td>
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<td>Chemotherapy</td>
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<tr>
<td>19</td>
<td>66</td>
<td>M</td>
<td>Surgery</td>
<td>15</td>
<td>NED</td>
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</tbody>
</table>

EWDA, extremely well-differentiated adenocarcinoma; F, female; ESD, endoscopic submucosal dissection; NED, no evidence of disease; AWD, alive with disease; M, male; mo, months after diagnosis; DOD, died of disease; NA, not available (cannot be assessed).

Table 2. Pathologic and endoscopic information of 19 cases of EWDA

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Tumor size, greatest dimension (mm)</th>
<th>Location</th>
<th>Endoscopic impression</th>
<th>Mucosal ulceration</th>
<th>Macroscopic finding</th>
<th>T/N</th>
<th>Distant metastasis</th>
<th>Lymphovascular invasion</th>
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<td>33</td>
<td>Body</td>
<td>SET</td>
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<td>EGC Ila</td>
<td>T1b/Nx</td>
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<td>Present</td>
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<td>2</td>
<td>NA</td>
<td>Body</td>
<td>SET</td>
<td>Absent</td>
<td>Bormann 1</td>
<td>T2/N+</td>
<td>Present</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>Body</td>
<td>SET</td>
<td>Absent</td>
<td>Bormann 1</td>
<td>T3/N+</td>
<td>Present</td>
<td>NA</td>
</tr>
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<td>50</td>
<td>Body</td>
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<td>T3/N1</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
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<td>25</td>
<td>Body</td>
<td>Slight elevation</td>
<td>Absent</td>
<td>Bormann 1</td>
<td>T4a/N0</td>
<td>Absent</td>
<td>Present</td>
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<tr>
<td>6</td>
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<td>Cardia</td>
<td>Typical Bormann 3</td>
<td>Present</td>
<td>Bormann 3</td>
<td>T3/N0a</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>Body</td>
<td>Slight elevation</td>
<td>Absent</td>
<td>AGC mimicking EGC type Ila</td>
<td>T3/N0</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>8</td>
<td>NA</td>
<td>Body</td>
<td>Bormann 4</td>
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<td>T3/N+</td>
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<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>Body</td>
<td>Slight elevation</td>
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<td>T3/N0</td>
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<td>Present</td>
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<td>Body</td>
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<td>SET</td>
<td>Present</td>
<td>Bormann 1</td>
<td>T3N1</td>
<td>Absent</td>
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</tr>
<tr>
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<td>33</td>
<td>Cardia</td>
<td>Typical Bormann 3</td>
<td>Present</td>
<td>Bormann 3</td>
<td>T2N0</td>
<td>Absent</td>
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</tr>
<tr>
<td>19</td>
<td>30</td>
<td>Body</td>
<td>Slight elevation</td>
<td>Absent</td>
<td>Bormann 4</td>
<td>T2N0</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

EWDA, extremely well-differentiated adenocarcinoma; SET, subepithelial tumor; EGC, early gastric cancer; NA, cannot be assessed; AGC, advanced gastric cancer.

*Macroscopic finding was classified according to World Health Organization criteria; *N+, clinically assessed nodal metastasis.
Macroscopic findings

Eighteen cases with available endoscopic data were studied for endoscopic impressions (Table 2, Fig. 2). In two-thirds of the cases, malignant nature was not easily recognizable even by an experienced gastroenterologist (JYA) (Endoscopically assessed Borrmann type 3, 3 of 18 [16.6%], Borrmann type 4, 2 of 18 [11.1%], and Borrmann type 2, 1 of 18 [5.6%]) (Fig. 2). Compared to usual gastric adenocarcinomas, EWDAs more frequently showed Borrmann type 4 and 1 lesions in surgically resected specimens (Borrmann type 4, 4 of 18 [22.2%] and Borrmann type 1, 4 of 18 [22.2%]) with less frequent ulcer formation. Also, ulcer proportions of Borrmann type 2 and 3 EWDAs were significantly lower than those of control group AGCs (mean value, 28.0% vs. 57.3%, p = .0048) (Supplementary Table S1, Supplementary Fig. S2).

Histologic assessment

Most of the cases were histologically gastric-type (13 of 19 [68.4%]), while the others were intestinal-type EWDAs (6 of 19 [31.6%]) (Table 3). Irregular glandular shape was universally present (19 of 19 [100%], pretreatment biopsy; 19 of 19 [100%], surgically resected specimen), and inharmonious glands were present in the majority (9 of 19 [47.4%], pretreatment biopsy; 11 of 19 [57.9%], surgically resected specimen). About half of the cases showed undulating mucin borders (11 of 19 [57.9%], pretreatment biopsy; 12 of 19 [63.1%], surgically resected specimen) or distended mucins (8 of 19 [42.1%], pretreatment biopsy; 9 of 19 [47.4%], surgically resected specimen). Hyperplastic polyps showed distended mucin in some (6 of 19 [31.6%]) but other descriptive findings of EWDA were not present. Intestinal metaplasias in the adjacent background mucosa tended to be more frequently observed in intestinal-type EWDAs than in gastric-type EWDAs (gastric-type EWDA, 4 of 13 [30.8%] vs. intestinal-type EDWA, 4 of 6 [66.7%], p = .238) (Table 3).

Histologic characteristics worth mentioning in each case were separately recorded (Fig. 1, Supplementary Fig. S1). Case No. 2 exhibited exceptionally large cells with mucin distention, which were approximately 20 times the size of mature lymphocytes. Case No. 3 showed deceptively benign-looking cells with small nuclei, simulating normal foveolar gland epithelium. The same cells were also noted in the patient’s omental biopsy indicating metastasis. In case No. 4, the neoplastic glands were cystically

Fig. 2. Endoscopic appearances of diagnostically difficult extremely well-differentiated adenocarcinoma cases. (A) A mucosal elevation with mostly intact mucosa simulating a subepithelial tumor is noted (case No. 1). (B) A slightly elevated lesion with vascular engorgement is noted at angle (case No. 6). (C) A slightly depressed lesion is observed in case No. 12. (D) Diffusely thickened gastric wall in the gastric body is observed (case No. 9). (E) A slightly elevated mass is identified in case No. 7. (F) Thickened gastric folds with vascular engorgement (endoscopically Borrmann type 4) are seen in case No. 13.
dilated progressing towards the serosa. Case No. 11 showed mucosal cystic glands invading muscularis propria with gastritis cystica profunda-like morphology.

The epicenters of EWDAs were in the deeper mucosa or submucosa with infrequent ulcer formation. The overlying mucosa was without ulcer but involved by frequent glandular cancerization (Fig. 1I), or focal mucosal openings leading to large, deeply seated glands (Fig. 1D). The latter pattern was reminiscent of lobular endocervical glandular hyperplasia of the uterine cervix. It was also remarkable that desmoplastic reactions were barely observed.

**Differentiation from non-neoplastic foveolar glands**

Regarding differential diagnosis between gastric-type EWDAs and hyperplastic foveolar glands, we focused on three histologic features: (1) irregular glandular shape, (2) irregular spacing of nuclei, and (3) disruption of four lines. Irregular glandular shape was present in all EWDAs in contrast to hyperplastic polyps (Table 3). Irregular spacing of the nuclei was a distinct feature of EWDAs, which referred to scattered nuclei not aligned to the basement membrane with disrupted polarity (Fig. 3). Four lines of the foveolar epithelium, formed by the apical mucin cap, base of the mucin cap, cytoplasm and nucleus [6] were disrupted or disappeared in EWDA glands.

**Molecular features**

All 19 cases were variably positive for gastric markers (Fig. 4, Supplementary Table S2). Positive MUC5AC and CDX-2 immunolabeling were closely associated with histological gastric- and intestinal-type EWDAs, respectively. MUC6 and MUC2 expressions were not prominent in both subtypes, unlike the immunohistochemical profile of usual type gastric adenocarcinomas with diffuse expression of either markers. On an immunohistochemical basis, 13 (68.4%) and six (31.6%) cases showed gastric- and intestinal-phenotypes, identical to the histological classifications (Supplementary Table S2). Although p53 and Ki-67 immunolabelings were mildly increased relative to normal foveolar epithelial cells, it was not distinct enough. Instead, their staining patterns were helpful because EWDAs showed diffusely increased staining patterns while benign glands showed locally increased staining in proliferative zones (Fig. 3E, F). C-erbB2 was equivocally expressed in two (case Nos. 2 and 13), but the silver in situ hybridization results were negative.

There also were a few cases with targeted cancer panel sequencing results (case Nos. 2, 3, and 9). A few notable mutations were found: NRAS G12D, STK11 Q220Pfs*38 (case No. 2), PTEN L108R and Y178C (case No. 3), and KRAS G12D (case No. 9) (Supplementary Table S3). Even though the functional significance of the PTEN L108R and Y178C mutations is not known,

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**Table 3. Histologic and immunohistochemical features of 19 cases of EWDA**

<table>
<thead>
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</table>

EWDA, extremely well-differentiated adenocarcinoma; I, inharmonious disproportionate glands; M, distended mucin; S, irregular glandular shape; U, undulating apical mucin border; NA, not available; IM, intestinal metaplasia.
the loss of PTEN protein expression in this tumor (Fig. 4D) suggested at least one of the two PTEN mutations be loss of function mutation.

Evaluation of pretreatment biopsies

One to eight pre-therapeutic endoscopic examinations were performed in 19 patients (median, 2; mean, 2.5). A total of 55

Fig. 3. Comparison between extremely well-differentiated adenocarcinoma (EWDA) and its mimickers. (A) Hyperplastic polyp shows an organized gland shape and aligned nuclei. (B) EWDA glands (case 4) show an irregular glandular shape and irregular nuclear spacing. (C) Normal foveolar epithelium displays regular nuclear spacing with maintenance of the “4 lines” (arrows): line 1, the gastric-type mucin vacuole; line 2, the base of the mucin vacuole; line 3, the cytoplasm; and line 4, the nuclei. (D) Case No. 6 shows large tumor cells with ample mucin, hyperchromatic nuclei, and disrupted four lines. (E) p53 expression is markedly increased in the tumor cells in contrast to the background foveolar epithelium of case No. 7 (p53 immunohistochemistry). (F) Ki-67 in normal foveolar epithelium shows increased expression only along the base of the crypts while the tumor glands (arrows) show a diffuse increase in case No. 8 (Ki-67 immunohistochemistry).
tissue biopsies from 19 patients were available for review. The original diagnoses were ‘adenocarcinoma’ or ‘suspicious for adenocarcinoma’ in 46 biopsies (46 of 55, 84%). Retrospective review of the remaining 11 specimens revealed that 10 of them were initially misdiagnosed (Supplementary Table 4).

**DISCUSSION**

EWDA consists of bland-looking malignant cells which is difficult to diagnose and less discussed in the literature. In our study, EWDA were deep-seated tumors in the body of the stomach with less ulcer formation. It was also notable that endoscopically slightly elevated lesions or subepithelial tumor-like lesions were common, which is rarely suspected for malignancy by endoscopists [7,8]. Four histologic features including irregular glandular shape, undulating apical mucin border, and inharmonious glands and distended mucin were key histologic features of EWDA. In addition, irregular nuclear spacing and disruption of the four lines were helpful in discriminating of gastric-type EWDA from intestinal-type EWDA. [496] Lobular endocervical glandular hyperplasia-like glands in the submucosa, also noted in other studies, were frequently observed accounting for frequent mucosal sparing of EWDA [1,2]. Those findings suggest that some of the EWDA cases might have been originated from a deeper part of the mucosa.

Gastric-type EWDA are especially cryptic because it simu-
lates hyperplastic foveolar glands, and in many circumstances the distinction should be made in small gastric forceps biopsies. Undulating apical mucin border, along with irregular glandular shape, irregular nuclear spacing, and the disruption of four lines of foveolar epithelium, should strongly suggest EWDA especially in ambiguous endoscopic settings. Mucin distension, although present in many EWDA, was sometimes seen in hyperplastic polyps and only marked mucinous distention sizing more than ×20 that of adjacent lymphocytes would help in differential diagnoses. Inharmoniously large glands in EWDA, as in our cases, also have been described in the literature [1,2], so atypically large or distinct glands should also raise concern for EWDA. Furthermore, even though intestinal metaplasias were rare adjacent to gastric-type EWDA, its pathogenetic significance is yet to be discussed.

One helpful immunohistochemical marker might be Ki-67, because the labeling index was randomly increased in the carcinoma cells in contrast to reactive lesions with only basal cryptal increase, consistent with the findings by Niimi et al. [9]. Another useful marker might be p53 because its expression was increased against the background gastric foveolar epithelium in EWDA [9], although null- or diffuse-type mutation pattern p53 immunolabeling [10] was not identified. Treatment-wise, C-erbB2 expression or actionable genetic alterations were not found in any. Further studies for the therapeutic targets are recommended in the future.

One of the limitations to our study is that the control group of AGC cases were retrieved from a different time period, due to the extreme rarity of EWDA cases. Furthermore, systematic quantitative analyses could not be performed because our study was focused on the morphologic diagnosis of very rare EWDA cases. Also, selection bias towards AGCs occurred because unequivocally metastatic cases were selectively included in the study design. The referral bias as a tertiary medical institution also contributed. Furthermore, NGS results are available only in a small portion of our cases. Patients lost to follow-up also posed problems because prognostic data could not be gathered sufficiently. We suggest that proper epidemiologic data of this rare neoplasm be assessed by a prospective, multi-center study in the future.

In conclusion, EWDA were endoscopically ambiguous elevated tumors with an undermining growth pattern and few ulcer. Mucosal glandular cancerization and submucosal lobular endocervical glandular hyperplasia-like growth pattern were frequently observed histologic patterns. The microscopic features such as irregular glandular shapes, inharmonious glands, glands with ample mucin distension, and undulating apical border were frequently observed in EWDA. For the timely diagnosis of these deeply seated tumors, generous forceps biopsies are recommended.

Supplementary Information
The Data Supplement is available with this article at https://doi.org/10.4132/jptm.2021.10.12.

Ethics Statement
Our study was approved by the Institutional Review Board of the Asan Medical Center (#2021-0485) with a waiver of the requirement for informed consent.

Availability of Data and Material
The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Code Availability
Not applicable.

ORCID
Jongwon Lee https://orcid.org/0000-0003-3057-7874
In-Seob Lee https://orcid.org/0000-0003-3099-0140
Ji Yong Ahn https://orcid.org/0000-0002-0030-3744
Young Soo Park https://orcid.org/0000-0001-5389-4245
Jihun Kim https://orcid.org/0000-0002-8694-4365

Author Contributions
Conceptualization: JL, JK, YSP, ISL, JYA. Data curation: JK, JL. Methodology: JL, JK, YSP, ISL, JYA. Writing—original draft: JL, JK. Writing—review & editing: JK, JL. Approval of final manuscript: all authors.

Conflicts of Interest
The authors declare that they have no potential conflicts of interest.

Funding Statement
No funding to declare.

References
6. Waters KM, Salimian KJ, Voltaggio L, Montgomery EA. Refined criteria for separating low-grade dysplasia and nondysplastic Barrett esophagus reduce equivocal diagnoses and improve prediction...
**Supplementary Table S1. Ulcer proportions of EWDAs and 17 AGCs from 2010**

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<tr>
<th>EWDA case No.</th>
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<th>Ulcer proportion (%)</th>
<th>Bormann type</th>
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EWDA, extremely well-differentiated adenocarcinoma; AGC, advanced gastric cancer. <sup>a</sup>Area of ulcer and tumor quantified with 2×2 mm<sup>2</sup> grid.
## Supplementary Table S2. Immunohistochemical features of 19 cases of EWDA

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EWDA, extremely well-differentiated adenocarcinoma.

\(^a\)C-Erb B2 grading followed the guidelines for HER2 testing issued by the College of American Pathologists; \(^b\)p53 grading, 1+: 10%~1/3; 2+, 1/3~2/3; 3+, >2/3.
EXTREMELY WELL DIFFERENTIATED CARCINOMA

**Supplementary Table S3.** Mutation profiles of the cases with available next-generation sequencing data

<table>
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<th>Stage</th>
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<td>STK11 Q220Pfs*38 mutation</td>
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</tr>
<tr>
<td></td>
<td>High ERBB3 amplification (~ 12 copies)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Strong MDM2 amplification (~ 35 copies)</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>PTEN mutations (L108R, Y178C)</td>
<td>Gastric-type</td>
<td>T3N⁺⁺M⁺⁺</td>
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<td>RET R79Q mutation</td>
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<td>9</td>
<td>KRAS G12D mutation</td>
<td>Intestinal-type</td>
<td>T3N0Mx</td>
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<tr>
<td></td>
<td>MYC amplification (16 copies)</td>
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aN⁺, nodal metastasis clinically assessed; bM⁺, distant metastasis clinically assessed.
### Supplementary Table S4. Retrospective comparative analysis of misdiagnosed pretreatment biopsies

<table>
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<th>Revised diagnosis</th>
<th>Reasons for missed diagnoses</th>
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<td>Hyperplastic foveolar epithelium showing dysplasia</td>
<td>Gastric-type EWDA</td>
<td>Mistaken as hyperplastic foveolar epithelium</td>
</tr>
<tr>
<td>3</td>
<td>A few atypical hyperplastic gland clusters, favor reactive</td>
<td>A few atypical hyperplastic gland clusters, suggestive of adenocarcinoma well differentiated</td>
<td>Mistaken as hyperplastic foveolar epithelium</td>
</tr>
<tr>
<td></td>
<td>Markedly hyperplastic atypical gastric foveolar glands with mild nuclear atypia,</td>
<td>Markedly hyperplastic atypical gastric foveolar glands with mild nuclear atypia,</td>
<td>Mistaken as hyperplastic foveolar epithelium</td>
</tr>
<tr>
<td></td>
<td>favor Menetrier's disease</td>
<td>suggestive of adenocarcinoma, well differentiated</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Atypical glandular proliferation</td>
<td>Gastric-type EWDA</td>
<td>Mistaken as foveolar epithelium</td>
</tr>
<tr>
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<td>Chronic active gastritis, marked, with intestinal metaplasia, regenerating atypia and</td>
<td>Atypical fundic type glandular proliferation with structural atypia,</td>
<td>Mistaken as regeneration atypia</td>
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<td></td>
<td>scar</td>
<td>suggestive of adenocarcinoma, well differentiated</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Atypically dilated glands in erosive background</td>
<td>Adenocarcinoma, very well differentiated</td>
<td>Mistaken as dilated foveolar glands</td>
</tr>
<tr>
<td>12</td>
<td>Atypical metaplastic glands in erosion, favor reactive</td>
<td>Gastric-type EWDA</td>
<td>Mistaken as metaplastic glands</td>
</tr>
<tr>
<td></td>
<td>Dilated benign-looking glands in thick disorganized muscularis mucosa,</td>
<td>Gastric-type EWDA</td>
<td>Mistaken as gastritis cystica profunda</td>
</tr>
<tr>
<td></td>
<td>suggestive of gastritis cystica profunda</td>
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</tr>
<tr>
<td>13</td>
<td>Chronic gastritis, mild with atrophy and foveolar epithelial hyperplasia</td>
<td>Gastric-type EWDA</td>
<td>Mistaken as hyperplastic foveolar epithelium</td>
</tr>
</tbody>
</table>

EWDA, extremely well-differentiated adenocarcinoma.
Supplementary Fig. S1. (A) Inharmonious disproportionate glands (arrows) are shown among the background foveolar epithelium in case No. 19 (B) Distended mucin of gastric-type extremely well-differentiated adenocarcinoma (EWDA) glands (arrows) is noted in case No. 6. (C) Irregularly distorted glandular shape of the EWDA (arrows) is shown with normal foveolar epithelium (arrowheads) in case No. 18. (D) EWDA glands demonstrate a wobbly, undulating apical mucin border (arrows) adjacent to the normal foveolar glands (arrowheads) in case No. 8.
Supplementary Fig. S2. (A) A Borrmann type 3 extremely well-differentiated adenocarcinoma (EWDA) (case No. 10) is found in the body of the stomach. (B) A case of EWDA is quantified with 2-mm grid. Area of ulceration (orange) and area of tumor (blue) are delineated (case No. 10). (C) A Borrmann type 3 advanced gastric cancer is the antrum of stomach. (D) Both area of ulceration (orange) and area of tumor (blue).
Fatty acid synthetase expression in triple-negative breast cancer

Jin Hee Park1, Hye Seung Han1, So Dug Lim1, Wook Youn Kim1, Kyoung Sik Park2,
Young Bum Yoo2, Seung Eun Lee1, Wan-Seop Kim1

Departments of 1Pathology and 2Surgery, Konkuk University Medical Center, Konkuk University School of Medicine, Seoul, Korea

Background: Triple-negative breast cancer (TNBC) has a relatively poor prognosis. Research has identified potential metabolic targets, including fatty acid metabolism, in TNBC. The absence of effective target therapies for TNBC led to exploration of the role of fatty acid synthetase (FASN) as a potential target for TNBC therapy. Here, we analyzed the expression of FASN, a representative lipid metabolism-related protein, and investigated the association between FASN expression and Ki-67 and the programmed death ligand 1 (PD-L1) biomarkers in TNBC.

Methods: Immunohistochemical expression of FASN was analyzed in 166 patients with TNBC. For analytical purposes, patients with 0–1+ FASN staining were grouped as low-grade FASN and patients with 2–3+ FASN staining as high-grade FASN.

Results: FASN expression was observed in 47.1% of TNBC patients. Low and high expression of FASN was identified in 75.9% and 24.1%, respectively, and no statistically significant difference was found in T category, N category, American Joint Committee on Cancer stage, or recurrence rate between the low and high-FASN expression groups. Ki-67 proliferation level was significantly different between the low and high-FASN expression groups. FASN expression was significantly related to Ki-67 as the level increased. There was no significant difference in PD-L1 positivity between the low- and high-FASN expression groups. Conclusions: We identified FASN expression in 166 TNBC patients. The Ki-67 proliferation index was positively correlated with FASN level, indicating higher proliferation activity as FASN increases. However, there was no statistical association with PD-L1 SP142, the currently FDA-approved assay, or FASN expression level.

Key Words: Triple-negative breast neoplasms; FASN; Immunohistochemistry; Ki-67; PD-L1 SP142

Received: September 29, 2021 Revised: October 20, 2021 Accepted: October 27, 2021

Corresponding Author: Seung Eun Lee, MD, PhD, Department of Pathology, Konkuk University Medical Center, Konkuk University School of Medicine, 120-1 Neungdong-ro, Gwangjin-gu, Seoul 05030, Korea
Tel: +82-2-2030-5644, Fax: +82-2-2030-5629, E-mail: 20150063@kuh.ac.kr

Corresponding Author: Wan-Seop Kim, MD, PhD, Department of Pathology, Konkuk University Medical Center, Konkuk University School of Medicine, 120-1 Neungdong-ro, Gwangjin-gu, Seoul 05030, Korea
Tel: +82-2-2030-5642, Fax: +82-2-2030-5629, E-mail: wskim@kuh.ac.kr

Triple-negative breast cancer (TNBC) is defined by a loss of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) [1]. TNBC accounts for about 15% of all breast cancers [2]. TNBC has a relatively poor prognosis compared to non-TNBC patients, resulting from a lack of specific treatment methods such as hormone treatment or targeted treatment other than non-specific chemotherapy [3]. TNBC is a heterogeneous group of tumors with different molecular drivers and prognosis, clinical outcomes, and responses to therapy [4]. Various inhibitors, including poly (ADP-ribose) polymerase (PARP), growth factor, Scr, mammalian target of rapamycin (mTOR), and phosphoinositide 3-kinase (PI3K) inhibitors, are intended to increase treatment effectiveness [5]. Based on the high level of programmed death ligand 1 (PD-L1) expression in TNBC, chemotherapy such as arezolizumab and nab-paclitaxel has been approved by the Food and Drug Administration (FDA) as the first-line treatment in breast cancer patients with locally advanced and metastatic TNBC [6]. Atezolizumab plus nab-paclitaxel-treated patients are linked to improved clinical outcome [7].

The study of metabolic pathways in TNBC is also active, and studies [8] have shown that potential metabolic targets of TNBC include glucose metabolism, fatty acid metabolism, glutamine metabolism, and serine metabolism. Gong et al. [9] identified three metabolic pathway-based TNBC subtypes with distinct molecular features and sensitivities to various metabolic inhibitors, and it was shown that inhibition of lactate dehydrogenase could enhance the anti–programmed death-1 immunotherapy
response in a certain TNBC subtype. Cha et al. [10] identified lipid metabolism–related proteins in breast cancer. Of them, fatty acid synthetase (FASN) is a lipid-producing enzyme that is expressed at low level in normal human tissues but is reported to be highly expressed in breast, colon, prostate, and ovarian cancer [11-14]. The role of FASN as a prognostic factor in breast cancer has been published in previous studies [15,16].

However, few studies have identified the expression of FASN level in TNBC [15,16]. The absence of effective targeted therapy for TNBC and its poor prognosis led to exploration of the role of FASN as a potential target for TNBC therapy [17]. Here, we analyzed the expression of FASN, a representative lipid metabolism–related protein, and the association between FASN level and Ki-67 and PD-L1 expression in patients with aggressive TNBC breast cancer.

MATERIALS AND METHODS

Among ER- and HER-2–negative breast cancer patients, 166 patients who could be tracked and whose paraffin embedded tissue blocks were in good condition were enrolled in the study. Patient clinical records were used in a retrospective manner to identify clinical information including age, immunohistochemical study, TNM stage, recurrence, chemotherapy, and radiation therapy. We also wanted to quantify the degree of expression and correlate it with clinical information through immunostaining of FASN.

Patient selection and clinicopathologic evaluation

From January 2012 to December 2018, 166 TNBC patients who underwent primary breast cancer surgical resection at Konkuk University Medical Center (KUMC) Seoul, Korea, were analyzed. Clinicopathological information was obtained by reviewing medical records and hematoxylin and eosin (H&E)–stained sections. The TNBC histopathological data were determined by histological subtype, category T, category N, American Joint Committee on Cancer (AJCC) stage, and Bloom-Richardson histological grade.

Tissue microarray

All 166 H&E-stained slides were reviewed, and the most representative part of each was selected. Two 3 mm tissue cores derived from representative tumors of formalin-fixed paraffin-embedded tissue blocks were collected. An on-slide control tissue (tonsillar tissue) was used.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a Ventana Discovery XT automated slide stainer (Ventana Medical Systems, Tucson, AZ, USA), following antigen retrieval with Cell Conditioning 1 (CC1; citrate buffer pH 6.0, Ventana Medical System) at 37°C for 32 minutes. This was followed by a standard Ventana signal amplification and counterstaining with hematoxylin. Slides were mounted and examined by light microscopy. Appropriate positive and negative controls for IHC were included. We used anti-fatty acid synthase antibodies (Abcam, Cambridge, UK) and SP142 antibodies (Ventana, Mannheim, Germany) in this study.

Interpretation of IHC results

All IHC markers were determined using light microscopy to assess the proportion and intensity of stained cells. In this study, we classified breast cancer phenotypes according to ER, PR, and HER-2 IHC results and Ki-67 [18]. ER and PR positivity were used to define a cutoff value of 1% positively-stained nuclei [19]. HER-2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists guidelines [20]. HER-2 expression was considered positive when strong (3+) membranous staining was observed, whereas cases with scores of 0 to 1+ were considered negative [10]. The results of immunohistochemical staining for lipid metabolism–related proteins were scored as previously described [21]. Briefly, FASN staining was considered positive when > 10% of the tumor cells were stained, and the intensity was scored from 0 to 3. For analytical purposes, patients with 0–1+ FASN staining were grouped as low-grade FASN and patients with 2–3+ FASN staining as high-grade FASN [15]. SP142 score was assessed as previously described [6].

Statistical analysis

Data analysis was performed using SPSS ver. 20.0 (IBM Corp., Armonk, NY, USA) for Windows. Chi-square and Fisher’s exact tests were used to assess continuous and categorical variables, respectively. Statistical significance was assumed at a p-value < .05. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate the time interval to tumor metastasis and survival duration. A Cox proportional hazards model was used to assess the risk factors of shorter disease-free survival and overall survival.
RESULTS

Clinicopathological characteristics of TNBC

A total of 166 TNBC patient results were analyzed in this study, and the clinicopathological characteristics are presented in Table 1. The median age at diagnosis was 51 years (range, 28 to 83 years). The major histologic type of TNBC in KUMC was invasive ductal carcinoma (92.8%), with eight carcinomas with medullary features, three pleomorphic carcinomas, and one metaplastic carcinoma. About 85% of the cases were histologic grade 3, and 66% of the cases were AJCC stage 2. Of these 166 patients, 10 (6.0%) received neoadjuvant chemotherapy, 154 (92.8%) received adjuvant chemotherapy, and 140 (84.3%) received radiation therapy. Among the 166 patients, 26 (15.7%) recurred during follow-up observation. The median time until recurrence was 13.5 months.

Table 1. Clinicopathological characteristics of 166 patients with TNBC

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>51 (28–83)</td>
</tr>
<tr>
<td>Age (yr) ≤ 50</td>
<td>81 (48.8)</td>
</tr>
<tr>
<td>Age (yr) &gt; 50</td>
<td>85 (51.2)</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
</tr>
<tr>
<td>IDC, NOS</td>
<td>154 (92.8)</td>
</tr>
<tr>
<td>Carcinoma with medullary feature</td>
<td>8 (4.8)</td>
</tr>
<tr>
<td>Pleomorphic carcinoma</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>Metaplastic carcinoma</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>T category</td>
<td></td>
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<tr>
<td>1</td>
<td>66 (39.8)</td>
</tr>
<tr>
<td>2</td>
<td>88 (53.0)</td>
</tr>
<tr>
<td>3</td>
<td>11 (6.6)</td>
</tr>
<tr>
<td>4</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>N category</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>109 (65.7)</td>
</tr>
<tr>
<td>1</td>
<td>40 (24.1)</td>
</tr>
<tr>
<td>2</td>
<td>9 (5.4)</td>
</tr>
<tr>
<td>3</td>
<td>4 (2.4)</td>
</tr>
<tr>
<td>NA</td>
<td>4 (2.4)</td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>24 (14.5)</td>
</tr>
<tr>
<td>3</td>
<td>142 (85.5)</td>
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<tr>
<td>AJCC stage</td>
<td></td>
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<tr>
<td>1</td>
<td>33 (19.9)</td>
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<tr>
<td>2</td>
<td>110 (66.3)</td>
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<tr>
<td>3</td>
<td>19 (11.4)</td>
</tr>
<tr>
<td>N/A</td>
<td>4 (2.4)</td>
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<tr>
<td>Neoadjuvant CTx</td>
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</tr>
<tr>
<td>Yes</td>
<td>10 (6.0)</td>
</tr>
<tr>
<td>No</td>
<td>156 (94.0)</td>
</tr>
<tr>
<td>CTx</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>154 (92.8)</td>
</tr>
<tr>
<td>No</td>
<td>12 (7.2)</td>
</tr>
<tr>
<td>RTx</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>140 (84.3)</td>
</tr>
<tr>
<td>No</td>
<td>26 (15.7)</td>
</tr>
<tr>
<td>Recur</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>26 (15.7)</td>
</tr>
<tr>
<td>No</td>
<td>140 (84.3)</td>
</tr>
</tbody>
</table>

Values are presented as median (interquartile range) or number (%). TNBC, triple-negative breast cancer; IDC, invasive ductal carcinoma; NOS, not otherwise specified; NA, not applicable; AJCC, American Joint Committee on Cancer; CTx, chemotherapy; RTx, radiation therapy.

Correlation with immunohistochemical study of Ki-67

The Ki-67 proliferation level was statistically different between the FASN low and high expression groups, with median Ki-67 levels of 60% (range, 40% to 80%) and 73% (range, 55% to 87%), respectively, using the Mann-Whitney U test (p = .003) (Table 3). The Spearman correlation coefficient was 0.257, indicating weak correlation between Ki-67 proliferation level and FASN expression level (p = .001).

Correlation with PD-L1 SP142 IHC assay

Two pathologists independently observed and agreed to the
final score values. PD-L1 expression was assessed using the PD-L1 SP142 assay. Of the 166 cases, 94 available cases were used to perform IHC for PD-L1 SP142. At the 1% cutoff value, 52.6% (50/94) of cases were positive for PD-L1 SP142 in infiltrating immune cells (ICs). We analyzed the correlation between FASN expression level and percentage of PD-L1 SP142 positive infiltrating ICs, but no correlation was identified. There was no statistically significant PD-L1 SP142 positivity between the low- and high-FASN expression groups. A representative case of SP142 expression in the expression of high FASN was confirmed (Fig. 2).

**DISCUSSION**

TNBC has no available special treatment other than chemotherapy after surgery, so early detection of recurrence through short-term follow-up observations will help patients with treatment and prognosis [22]. The development of interval breast cancer within the time interval between screening examinations has more adverse biological features, poorer survival outcomes, and is more highly associated with TNBC [23]. TNBC has high prevalence in young women under 50 and is more common in African Americans [24]. TNBC is known to have relatively poor prognosis compared to non-triple-negative breast cancer, most likely resulting from lack of special treatment methods such as hormone treatment or targeted treatment other than non-specific chemotherapy [25].

The TNBC subtype was classified into four distinct types by Burstein et al. [26] and is being actively studied because of different prognoses and target therapies. Clinical results and treatment responses have changed depending on classification [27]. These inherent differences related to TNBC sub-classification have resulted in a renewed effort to identify driver mutations and more appropriate targeted treatment [28]. For potential therapy, activated progesterone receptor, platinum, tyrosine kinase inhibitor, PI3K/mTOR inhibitor, anti-PD-L1 inhibitors, and/or androgen receptor targeted PI3K inhibitors are thought to be
survival rate [29]. Therefore, these treatments are being attempted in TNBC patients to identify whether they can benefit from a more appropriate and targeted treatment. We performed IHC for FASN, a representative lipid metabolism–related protein, as a candidate for a potential specific target in TNBC.

FASN is a key lipogenic enzyme and is known to be overexpressed in various human cancers [30]. FASN is a lipid-producing enzyme that is expressed at low levels in normal human tissues but is reported to be highly expressed in breast, colon, prostate, ovarian, and endometrial cancers [11-14]. It has been hypothesized that useful indicators of prognosis can be found based on differences in the degree of expression between normal and cancer tissues, and patients with high FASN expression level have been reported to show lower disease-free survival and decreased survival rate [31]. High FASN expression was identified in 45% of TNBC cases [15] and high-FASN groups have been significantly associated with positive nodal status [15,16]. Therefore, FASN has emerged as a potential target, and FASN inhibitors are being evaluated in clinical trials [32]. First-generation (e.g., orlistat and cerulenin) and next-generation (TVB-3166 and TVB-2640) FASN-targeting drugs have been developed [33]. Adipophilin (ADP) and FASN are two lipid metabolism–related proteins of clinicopathological relevance for IHC expression in salivary duct carcinoma. ADP-positive expression was associated with the presence of prominent nuclear pleomorphism, high Ki-67, and poor prognosis [30].

In this study, we evaluated the association of FASN expression between clinicopathological features, including PD-L1 SP142 expression, and further evaluated its usefulness as a biomarker in TNBC. FASN expression was observed in 47.1% (95/166) of TNBC cases [15]. Low-FASN groups have been significantly associated with positive nodal status [15,16]. Therefore, FASN has emerged as a potential target, and FASN inhibitors are being evaluated in clinical trials [32]. First-generation (e.g., orlistat and cerulenin) and next-generation (TVB-3166 and TVB-2640) FASN-targeting drugs have been developed [33]. Adipophilin (ADP) and FASN are two lipid metabolism–related proteins of clinicopathological relevance for IHC expression in salivary duct carcinoma. ADP-positive expression was associated with the presence of prominent nuclear pleomorphism, high Ki-67, and poor prognosis [30].

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Ki-67 IHC is used as a proliferation marker in breast cancer and is a low-cost measurement method suitable for extensive use in clinical practice [34]. Higher Ki-67 level is associated with increased early recurrence and aggression, resulting in a lower patient survival rate [35]. In a Japanese study, when Ki-67 was greater than 10%, it was related with a high recurrence rate and low survival rate [36]. The Breast Cancer Working Group proposed guidelines for Ki-67 assessment in breast cancer and use of this potentially important marker based on current evidence [37]. Less than 5% of patients do not require chemotherapy, and more than 30% show chemotherapy indication. The ER negative case is referred to as insufficient evaluation for Ki-67 [37]. Another group reported that high Ki-67 expression (cutoff value of 30%) was correlated with early recurrence in luminal B/HER-2 negative breast cancer [38]. TNBC patients in the high Ki-67
The majority of TNBC cases have high Ki-67 expression level (≥ 20%), which is used as a proliferation factor. In our study, 92.8% of cases had Ki-67 expression ≥ 20%. However, the Ki-67 proliferation level was statistically different between the FASN low and high expression groups. The median Ki-67 value of the low- and high-FASN expression groups was 60% (range, 40% to 80%) and 73% (range, 55% to 87%), respectively.

In 2019, the FDA-approved atezolizumab (TECENTRIQ, Genentech Inc.) treatment in combination with paclitaxel was approved for TNBC patients whose PD-L1 stained tumor-infiltrating ICs were over 1% intensity. PD-L1 is a cell membrane protein expressed in tumor cells and ICs. PD-L1 expression is increased in TNBC and is a positive predictor of immunotherapy. PD-L1 IHC using the VENTANA SP142 assay, the current FDA-approved assay, improved clinical outcome in atezolizumab plus nab-paclitaxel-treated patients. In our study, we investigated the association between PD-L1 and FASN expression level and unfortunately identified no association.

TNBC represents a small proportion of breast cancer subtypes but has the worst outcome. Although this study was based on data from a small number of TNBC cases and short-term follow-up surveys, we included 166 TNBC patients in the study. More research on the expression of FASN on TNBC and its potential inhibitors will help identify new target treatments. Further research is needed on how FASN correlates with other factors in TNBC. In addition, further studies are required with a larger number of TNBC patients to investigate the specific role of FASN in TNBC and the possibility for FASN inhibition as a target treatment for TNBC.

In conclusion, we showed FASN expression in TNBC patients and Ki-67 proliferation index to be positively correlated with FASN level, indicating higher proliferation activity as FASN increases. However, there was no statistical association with PD-L1 SP142, the currently FDA-approved assay, or FASN expression level.

**Ethics Statement**
The KUMC Institutional Review Board anonymized the archive data prior to the study and waived the requirement for prior consent (KUMC 2021-07-032).

**Availability of Data and Material**
The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

**Code Availability**
Not applicable.

**ORCID**
Jin Hee Park https://orcid.org/0000-0003-1277-7549
Hye Seung Han https://orcid.org/0000-0002-3591-9995
So Dug Lim https://orcid.org/0000-0003-2036-0313
Wook Youn Kim https://orcid.org/0000-0003-4024-8791
Kyoung Sik Park https://orcid.org/0000-0001-9806-9839
Young Bum Yoo https://orcid.org/0000-0003-0731-8807
Seung Eun Lee https://orcid.org/0000-0002-7459-0061
Wan-Seop Kim https://orcid.org/0000-0001-7704-5942

**Author Contributions**
Conceptualization: SEL, WSK. Data curation: KSP, YBY, JHP. Formal analysis: SEL, WYK. Funding acquisition: WSK. Investigation: JHP. Methodology: SEL. Supervision: WSK, HSH. Validation: SEL, SDL. Writing—original draft: JHP, SEL. Writing—review & editing: SEL, WSK, JHP. Approval of final manuscript: all authors.

**Conflicts of Interest**
The authors declare that they have no potential conflicts of interest.

Fig. 2. A representative case of SP142 (B) expression in the expression of high fatty acid synthase (FASN) (A) was confirmed.
Funding Statement
This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (Ministry of Science and ICT) (No. 2019M3E5D3073475).

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In December 2019, several patients in China were infected with a novel coronavirus known as severe acute respiratory syndrome coronavirus 2, the etiological agent underlying acute respiratory disease named coronavirus disease 2019 (COVID-19) [1]. Based on clinical and radiologic data, lung injury is one of the most common complications of COVID-19. In about 20% of COVID-19 patients, acute lung injury (ALI) rapidly progresses into acute respiratory distress syndrome (ARDS) [2]. ARDS development includes acute, subacute, and chronic phases [3]. The acute phase is mainly characterized by alveolar and interstitial edema, endothelial and epithelial damage, and aggregations of inflammatory cells and red blood cells (RBCs) in the alveoli. The predominant changes in the subacute phase are pulmonary edema clearance, type II alveolar pneumocyte hyperplasia, fibroblastic proliferation, and collagen deposition [3]. The chronic phase of ARDS involves infiltrations of alveolar macrophages in the alveoli and increased fibrosis. The bleomycin-induced lung injury model is a well-characterized model of pulmonary damage, edema, inflammation, and eventual fibrosis that closely resembles the ARDS development pathway [4]. The bleomycin mouse model is an excellent research tool to investigate ARDS due to strong similarities regarding the cells, mediators, and signaling pathways that contribute to pathogenesis.

Bleomycin, generated by the bacterium Streptomyces verticillus, is a glycosylated linear non-ribosomal peptide antibiotic. Since bleomycin has potent antitumorigenic properties, it is used as a chemotherapeutic agent against many cancers, including squamous cell carcinomas of the cervix, esophagus, and head and neck;

**Background:** Acute respiratory distress syndrome (ARDS) is one of the most common complications in coronavirus disease 2019 patients suffering from acute lung injury (ALI). In ARDS, marked distortion of pulmonary architecture has been reported. The pulmonary lesions in ARDS include hemodynamic derangements (such as alveolar edema and hemorrhage), vascular and bronchiolar damage, interstitial inflammatory cellular aggregations, and eventually fibrosis. Bleomycin induces ARDS-representative pulmonary damage in mice and rats; therefore, we used bleomycin model mice in our study. Recently, Toll-like receptor 9 (TLR9) was implicated in the development of ARDS and ALI. **Methods:** In this study, we evaluated the efficiency of a TLR9 blocker (ODN2088) on bleomycin-induced pulmonary damage. We measured the apoptosis rate, inflammatory reaction, fibrosis in bleomycin- and bleomycin+ODN2088-treated mice. **Results:** Our results showed a significant amelioration in bleomycin-induced damage to pulmonary architecture following ODN2088 treatment. A marked decrease in pulmonary epithelial and endothelial apoptosis rate as measured by cleaved caspase-3 expression, inflammatory reaction as indicated by tumor necrosis factor α expression, and pulmonary fibrosis as demonstrated by Van Gieson staining and α-smooth muscle actin immunohistochemistry were observed following ODN2088 treatment. **Conclusions:** All these findings indicate that blocking downstream TLR9 signaling could be beneficial in prevention or mitigation of ARDS through hemodynamic derangements, inflammation, apoptosis, and fibrosis.

**Key Words:** TLR9; ODN2088; Bleomycin; Acute respiratory distress syndrome; Fibrosis
germ cell tumors; and both Hodgkins and non-Hodgkins lymphoma [5-7]. Bleomycin causes direct cellular damage by disrupting DNA strands, creating free radicals, and inducing oxidative stress [8,9]. Upon damage, the affected cells excrete uric acid and many other factors that initiate aggradation of alveolar macrophages and release of pro-inflammatory cytokines. During the first week after bleomycin administration, marked elevation of pro-inflammatory cytokines (tumor necrosis factor α [TNF-α], interleukin [IL]-1, and IL-6) was reported. Upregulation of profibrotic markers (transforming growth factor β1 [TGF-β1], fibronectin, and collagen) was seen by day 14. By the second to third weeks, patchy fibrotic areas with extracellular matrix (ECM) deposition, mainly in the forms of fibronectin and collagen I, are evident in pulmonary tissue [10].

Toll-like receptors (TLRs) have a vital role in the innate immune system [11-13]. Hyperactivation of TLRs disrupts immune system homeostasis, resulting in extensive pro-inflammatory cytokine production that has a key role in many autoimmune and inflammatory diseases [14]. Among other TLRs, TLR9 was found to promote the release of pro-inflammatory mediators such as TNF-α and IL-1β in ALI and is implicated in several chronic inflammatory pulmonary conditions [15]. Recent studies showed that TLR9 contributes to the development and worsening of ARDS and ALI in COVID-19 patients [16-18]. Furthermore, TLR9 induction was found to activate blood coagulation in humans and mice [19,20]. All these findings suggest that blocking the TLR signaling pathway could be an effective therapeutic strategy for mitigating the clinical presentation of ARDS and ALI in COVID-19 patients.

CpG-oligodeoxynucleotide ODN is an unmethylated, short, single-stranded synthetic DNA molecule that acts as a TLR9 ligand [21]. Activation of TLR9 using the ODN ligand promotes neutrophilic infiltration, pulmonary edema, and systemic inflammation [15,16]. The oligonucleotide ODN2088 (a DNA sequence) inhibits the activity of TLR9 and its downstream signaling effectors by disturbing the co-localization of unmethylated CpG di-nucleotides with TLR9 [22]. A previous study showed that ODN2088 inhibits the liver inflammation and fibrosis induced by CCl4 [23]. In the present study, a bleomycin-induced lung injury mouse model was used to evaluate the impact of TLR9 inhibition by ODN2088 on the lung damage, inflammation, and fibrosis commonly seen in ARDS in COVID-19 patients.

MATERIALS AND METHODS

Chemicals

CpG ODN2088 was purchased from Invitrogen (Carlsbad, CA, USA). ODN 2088 is a TLR9 antagonist with 15 base pairs (5'-tcctggcgggagaaggt-3'). Bleomycin sulfate (Celon Laboratories Pvt. Ltd., Hyderabad, India) was dissolved in sterile saline solution for injection.

In vivo experimental design

Swiss Albino mice were purchased from the Facility of Laboratory Animals at the Facility of Veterinary Medicine, Benha University, Egypt. All animal experiments were conducted according to protocols approved by the Benha University Committee of Bioethics in Egypt. Mice were maintained according to the National Institutes of Health standards established in the "Guidelines for the Care and Use of Laboratory Animals." Forty adult 6–8-week-old male Swiss Albino mice (22–30 g) were used in this study. The mice were subdivided equally into four groups. In the first group (control), mice were injected intraperitoneally (IP) with saline (vehicle). In the second group (bleomycin), mice were injected with bleomycin IP (2 mg/mouse) on days 1, 8, and 15. In the third group (ODN2088), the mice were injected IP with CpG ODN2088 (50 μg/20 g BW, re-suspended in endotoxin-free water) daily until the day of sacrifice. In the fourth group (bleomycin + ODN2088) (BLEO/ODN2088), the mice received both bleomycin (2 mg/mouse) and ODN2088 (50 μg/20 g BW).

Histopathology

The mice were euthanized after 30 days by cervical dislocation, and pulmonary tissue specimens were collected during necropsy. Lung specimens were fixed in 10% neutral-buffered formalin at room temperature for 72 hours, embedded in paraffin, cut in 4 μm sections, and stained with hematoxylin and eosin. Histological images of the slides were collected using a Nikon Eclipse E800 microscope (Melville, NY, USA) equipped with a camera and were analyzed using Fiji ImageJ software (ImageJ 1.51u, National Institutes of Health, Bethesda, MD, USA).

Van Gieson staining

Pulmonary tissue sections were washed with tap water and incubated in picrofuchsin solution (1% acid fuchs in aqueous saturated picric acid) for 2 minutes. Sections were dehydrated and mounted using DPX mounting agent (Thermo Fisher Scientific, Leicester, UK). Upon staining, collagen fibers appear pink to red, while muscle appears yellowish. The area of positive collagen or
elastin fibers was calculated compared to the total area of the tissue section.

**Immunohistochemistry**

The 5-μM-thick paraffin-embedded tissue sections were preheated and dewaxed in xylene followed by dehydration in ethanol. The tissue sections were incubated with antigen retrieval solution (Dako, Carpinteria, CA, USA) for 40 minutes using a steamer (Black & Decker HS1000, Newark, DE, USA) followed by a cooling step (for 30 minutes at room temperature). The tissue sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity and washed, and 10% bovine serum albumin was added to block non-specific immunoreactivity. The slides were incubated overnight with either anti-cleaved caspase-3 (1:400, Cat #Asp175, Cell Signaling Technology, Danvers, MA, USA), anti-α-SMA (1:400, NB501-19532, Novus, Centennial, CO, USA), or anti–α-smooth muscle actin (α-SMA; 1:50, clone 1A4, Dako) primary antibodies. On the next day, slides were washed three times and incubated with the corresponding biotinylated secondary antibody dilute in phosphate buffered saline (PBS) for 1 hour at 25°C, followed by three PBS washes. The slides were incubated with diaminobenzidine tetrahydrochloride solution for 10 minutes. The slides were washed three times in distilled water and counter stained with hematoxylin. Tissue sections were finally dehydrated using graded alcohols and xylene and then covered with a glass slip [24]. DAB staining (brown color) was recorded as a positive result. Positivity was counted as number of positive cells/number of total cells. ImageJ ver. 1.48 (National Institutes of Health, Bethesda, MD, USA) was used to calculate the percent positivity in all the pulmonary tissues.

**Statistical analysis**

All data collected are displayed as mean ± standard deviation. Student’s unpaired t-test was used to analyze the difference in positivity between bleomycin and BLEO/ODN2088 groups using GraphPad Prism ver. 6.03 (La Jolla, CA, USA). Differences denoted by p ≤ .05 were considered statistically significant.

**RESULTS**

**ODN2088 restored pulmonary architecture distorted by bleomycin**

In control mice, almost all the lung sections had normal pulmonary architecture with clear empty alveoli and thin interalveolar septa (Fig. 1A). The alveoli were lined with flat (type I pneumocytes) and cuboidal cells projecting into the alveolar lumen (type II pneumocytes). Patent bronchioles lined with simple columnar epithelium were also evident in most sections. Most lungs in ODN2088-treated mice exhibited normal histological appearance with thin interalveolar septa and patent alveoli. In a few lungs, mild alveolar pneumocyte hyperplasia was observed (Fig. 1B). Alveolar emphysema was seen occasionally in some pulmonary areas.

In bleomycin-treated mice, pulmonary architecture was severely distorted in a patchy pattern. Multifocal thickening of interalveolar septa and alveolar collapse were the most pronounced microscopic findings in all the lung sections. Meanwhile, most other alveoli were over-inflated (emphysematous). Alveolar septal thickening was mainly due to moderate to severe mononuclear inflammatory cell infiltration in the interalveolar space (Fig. 1C–E). Moderate type II pneumocyte hyperplasia with reactive atypia and fibroplasia was evident in several pulmonary areas. Focal alveolar histiocytosis characterized by accumulation of foamy macrophages in the alveoli was also prominent in some lung sections. Fine eosinophilic fibrin threads were seen in some alveoli. In many pulmonary sections, hyperplasia of the bronchiolar epithelium was reported. Some hyperplastic bronchioles had cytoplasmic vacuolation and prominent enlarged nuclei in their epithelia, while others suffered from necrosis of their lining epithelia with the presence of desquamated intraluminal cells (Fig. 1F–H). Inflammatory peribronchial cell aggregations and fibrosis were also reported in some foci (Fig. 1H–J). Congestion of peribronchiolar blood vessels and interalveolar capillaries, edema, and hemorrhage were the main vascular changes in bleomycin-treated group. There were extensive perivascular inflammatory cell infiltrates, including lymphocytes and macrophages, affecting some large pulmonary blood vessels along with extravasated RBCs in nearby alveoli (Fig. 1J). Fibrinoid necrosis was also observed in the walls of a few large pulmonary blood vessels in some sections (Fig. 1J). Other vascular alterations included disorganization and hypertrophy of tunica media, fibrous remodeling, and occasional pulmonary vein thrombosis (Fig. 1K). Multifocally, some alveoli were filled with extravasated RBCs, homogenous eosinophilic fluid, and siderophages. In some mice, pleura thickened by edema, fibrin deposition, and occasional infiltration of lymphocytes and macrophages and, rarely, neutrophils were seen (Fig. 1L).

Apparent amelioration of bleomycin-induced lung distortion, marked by distinct restoration of lung architecture, was noticed in the BLEO/ODN2088-treated group compared to the bleomycin-treated group. Most bronchiolar epithelia had mild goblet cell hyperplasia or minimal degenerative/necrotic changes (Fig. 1M). Most alveoli were devoid of fluids, RBCs, fibrin threads,
Fig. 1. Representative lung photomicrographs in control- (A), ODN2088- (B), bleomycin- (C–L), and bleomycin + ODN2088 (BLEO/ODN2088)–treated mice (M–O). (A) Normal respiratory bronchioli and alveoli in the control group. (B) Mild hyperplasia of alveolar pneumocytes in the lung of an ODN2088-treated mouse. (C–L) Lung sections of mice treated with bleomycin, showing focal to confluent, moderate, to severe aggregation of inflammatory cells in the interalveolar septa (C–E), hyperplastic bronchiolar epithelia (F), peribronchial inflammatory aggregations and necrosis of bronchiolar epithelia (G, H, arrowhead), peribronchiolar fibroplasia (I, arrow), perivascular (arrow) inflammatory cellular infiltration and intra-alveolar hemorrhage (J), thrombosis in a pulmonary blood vessel (K, arrowhead), and pleural thickening (L). (M–O) Lungs of BLEO/ODN2088-treated mice showing necrosis in a few bronchiolar epithelia (M), small focal inflammatory aggregation in the interstitial space (N), and congested interalveolar blood capillaries (O).
and inflammatory cells. There was no to mild thickening of interlobular septa due to mild mononuclear cell aggregations, congestion of interalveolar blood capillaries, and mild interlobular edema (Fig. 1N, O). Overinflation of alveoli with mild edema was occasionally seen.

**ODN2088 reduced bleomycin-induced pulmonary fibrosis**

Van Gieson staining and α-SMA immunohistochemistry were used to assess the antifibrotic effects of ODN2088 against pulmonary fibrosis induced by bleomycin. Van Gieson-stained sections demonstrated control and ODN2088-treated mice to have only a thin layer of pink-stained collagen fibers in the tunica adventitia of pulmonary blood vessels and bronchioles. In bleomycin-treated mice, on the other hand, multifocal areas of tightly packed pink-stained collagen fibers were seen around bronchioles and blood vessels (Fig. 2A, B). Multifocal red-stained collagen fibers were also seen in the interalveolar and interlobular spaces. The pulmonary tissues of the BLEO/ODN2088 group stained with Van Gieson stain were nearly similar to those of the control group (Fig. 2C). Compared to the bleomycin group, the area of Van Gieson-stained collagen fibers was significantly smaller in the BLEO/ODN2088-treated group (Fig. 2D).

**ODN2088 mitigated bleomycin-induced myofibroblast proliferation in pulmonary tissue**

To further confirm the findings of Van Gieson staining, we used α-SMA as an immunohistochemical marker for myofibroblast proliferation and fibrosis. There was very weak α-SMA immunos-
taining in the pulmonary tissues of control and ODN2088-treated mice. In bleomycin-treated mice, a marked increase in α-SMA protein expression (evidenced by brown DAB staining) was seen multifocally in the alveoli, interalveolar septa, and peribronchial connective tissue (Fig. 3A, B). ODN2088 reduced myofibroblast proliferation in the pulmonary tissues of the BLEO/ODN2088-treated group, demonstrated by a marked decline in α-SMA expression (Fig. 3C, D). Focal areas with mild α-SMA staining were seen in the interalveolar septa of some lungs in this group.

ODN2088 lowered the apoptosis rate in BLEO/ODN2088-treated pulmonary tissues

Staining for cleaved caspase-3 was used to assess the effect of ODN2088 on apoptosis caused by bleomycin. There was minimal cleaved caspase-3 expression in the control- and ODN2088-treated groups. Extensive damage to pulmonary tissues was seen in bleomycin-treated mice, where there was a marked increase in cleaved caspase-3 expression in many alveolar pneumocytes, bronchiolar epithelial cells, and vascular endothelial cells (Fig. 4A, B). ODN2088 mitigated the pulmonary toxicity of bleomycin in the BLEO/ODN2088 group. The ameliorative effects of ODN2088 were pronounced in bronchiolar epithelium and endothelial cells (Fig. 4C). Compared to the bleomycin group, there were significantly fewer cleaved caspase-3–positive cells in the BLEO/ODN2088-treated group (Fig. 4D).

![Fig. 3. Immunohistochemical photomicrographs (A–C) and statistical analysis (D) of α-smooth muscle actin (α-SMA) expression in the pulmonary tissues of bleomycin- (A, B) and bleomycin+ODN2088 (BLEO/ODN2088)–treated mice (C). Data in the bar graph (D) represent mean±standard deviation, and significant differences (p≤.05) are indicated by an asterisk.](https://patholtm.org/)

https://doi.org/10.4132/jptm.2021.12.27
ODN2088 mitigated the bleomycin-induced inflammatory reaction

To assess the potential inhibitory effects of ODN2088 on the bleomycin-induced inflammatory reaction, we evaluated TNF-α expression in pulmonary tissues. Null to mild TNF-α expression was seen in the pulmonary tissues of control and ODN2088-treated mice. In bleomycin-treated mice, extensive TNF-α expression was observed in alveolar macrophages and the inflammatory cellular aggregations in the interalveolar space and peribronchiolar, perivascular, and pleural connective tissue (Fig. 5A, B). ODN2088 markedly reduced TNF-α expression in pulmonary tissues in the BLEO/ODN2088-treated group. There was mild cellular TNF-α expression in the interalveolar septa and peribronchiolar connective tissue (Fig. 5C). In only one mouse, moderate to severe focal TNF-α expression was observed in the inflammatory cells in the interalveolar septa. The number of TNF-α positive cells was much lower in the BLEO/ODN2088-treated group compared to the bleomycin group (Fig. 5D).

**DISCUSSION**

Pulmonary damage is one of the most serious complications of diffuse lung diseases, such as idiopathic pulmonary fibrosis, interstitial lung disease, and ARDS [25-29]. Persistent pulmo-
Pulmonary damage has been shown to cause inflammation in the lungs, which eventually leads to pulmonary fibrosis [30]. Pulmonary fibrosis causes lung architecture distortion and excessive matrix deposition, which leads to respiratory insufficiency and death [31]. Many efforts are ongoing to alleviate the inflammatory process, lung injury, and pulmonary fibrosis to improve the quality of life in patients suffering from ARDS. There are several animal models that mimic the stages of pulmonary fibrosis in humans. In this research, we used bleomycin to induce lung fibrosis in mice since bleomycin-induced lung injury is a well-studied model of the changes seen in many pulmonary diseases, such as idiopathic pulmonary fibrosis, interstitial lung disease, and ARDS [26-29, 32]. Prior studies have indicated that ODN2088 is a potent anti-inflammatory agent that halts downstream TLR9 pro-inflammatory pathways [33]. Therefore, the potential efficacy of ODN2088 in preventing bleomycin-induced lung damage, inflammation, and fibrosis was investigated in this study. Our study found that blocking TLR9 with ODN2088 prevented lung fibrosis in bleomycin-treated mice by reducing lung injury and inflammation.

Bleomycin initiates lung injury by generating reactive oxygen species that cause subsequent DNA damage [6]. In addition, bleomycin promotes protein oxidation and lipid peroxidation
and induces an extreme inflammatory response and fibroblastic proliferation in animal models [34]. The fibrosis induced by bleomycin could be attributed to the cytokines secreted from alveolar macrophages following lung injury, such as TGF-β, IL-1, platelet-derived growth factor, and macrophage inflammatory protein-1 [35]. These cytokines promote the proliferation of myofibroblasts and stimulate the production of abnormal extracellular matrix, leading to fibrosis [36]. Consistent with prior studies on the bleomycin mouse model, the lungs of bleomycin-treated mice in our study showed activation of alveolar macrophages, intense inflammatory response, and fibroblastic and myofibroblastic proliferation, which mimic the stages of lung injury/fibrosis in humans [27,37].

TLRs play an important role in recognizing viruses and triggering the innate immune system [38]. TLR stimulation triggers downstream signaling cascades, resulting in the release of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1, and interferon type 1. It has been reported that, in severe cases of COVID-19, patients have marked elevation of inflammatory cytokines, such as IL-6, IL-8, IL-10, TNF-α, interferon-γ, IL-2, and IL-4 [39,40]. All these findings indicate that targeting TLRs could mitigate the clinical presentation, outcomes, and prognosis of COVID-19. ODN2088 is a murine TLR9 antagonist that has been shown to be potent in preventing hepatic inflammation and fibrosis in a CCl4-induced liver fibrosis mouse model [23,33]. In this study, we evaluated ODN2088 as a potential therapeutic agent for prevention of pulmonary injury, inflammation, and fibrosis.

Apoptosis has a vital role in initiating and sustaining fibrosis during the pathophysiology of interstitial lung disease [41]. The pathogenic mechanisms of bleomycin-induced lung fibrosis and idiopathic pulmonary fibrosis (IPF) involve induction of alveolar epithelial cell apoptosis through either the mitochondrial pathway or the caspase cascade linked to the Fas-FasL pathway [42,43]. Our results showed that ODN2088 greatly decreased the proportion of apoptotic bronchial cells, endothelial cells, and alveolar pneumocytes in bleomycin-treated mice, as represented by low cleaved caspase-3 immunostaining.

Recent studies attributed the lung fibrosis seen in COVID-19 cases to an excessive inflammatory response [44]. TNF-α, an important mediator of inflammation, triggers TGF-β1 and nuclear factor-κB and enhances proliferation and fibroblast differentiation, which ultimately lead to pulmonary fibrosis [45,46]. Previous research showed that a TNF-α antagonist alleviated bleomycin-induced pulmonary fibrosis in mice [47]. In this context, we evaluated ODN2088 as an anti-inflammatory agent against bleomycin-induced pneumonia. In this study, ODN2088 mitigated the inflammatory response in injured lungs, as evidenced by low TNF-α expression and a mild inflammatory response in the pulmonary tissues of BLEO/ODN2088-treated mice.

Prior studies emphasized that TLR9 is upregulated in IPF lungs, and CpG-ODN (a TLR agonist) promotes the differentiation of IPF-derived lung fibroblasts into myofibroblasts in vitro [48]. In addition, CpG-ODN was found to induce renal interstitial fibrosis in a mouse model of lupus nephritis [49]. Consistent with these previous studies, the present study showed the absence of pulmonary myofibroblasts in ODN2088-treated lung tissues compared to bleomycin-treated ones. Moreover, α-smooth actin immunostaining and Van Gieson staining were used to confirm the inhibition of myofibroblast differentiation and subsequent fibrosis. The α-smooth muscle actin expression level and the proportion of Van Gieson–stained collagen fibers were significantly lower in the lungs of BLEO/ODN2088-treated mice compared to those of bleomycin-treated mice. All these findings demonstrate that ODN2088 directly inhibits myofibroblast differentiation and development of pulmonary fibrosis induced by bleomycin.

Furthermore, our in vivo study showed a significant ameliorative effect in the lungs of mice given ODN2088 and bleomycin, as evidenced by a decrease in hemodynamic pulmonary lesions caused by bleomycin, such as microvascular damage, pulmonary edema, thrombosis, and hemorrhage. In coronavirus-infected patients, diffuse alveolar damage with serious pulmonary thrombosis and vascular injury has been documented [50], indicating that targeting TLR9 can improve the clinical presentation of ARDS, seen in some COVID-19 patients.

In conclusion, our study demonstrated that ODN2088 was potent in prevention of bleomycin-induced pulmonary injury, inflammation, and fibrosis. ODN2088 markedly diminished alveolar and vascular damages in pulmonary tissues and decreased the production of pro-inflammatory cytokines, inflammatory cellular aggregations, and myofibroblastic proliferation. These findings indicate that blocking TLR9 could be beneficial to treatment of severe acute respiratory syndrome, Middle East respiratory syndrome, and COVID-19 as a novel therapeutic modality to prevent pulmonary injury, fibrosis, and associated hemodynamic abnormalities, especially in the early stages.

**Ethics Statement**

All animal work was approved by the Bioethics Committee of Benha University. All in vivo experiments were conducted after the approved by Animal Care and Use Committee of Benha University. Animal welfare and comfort was emphasized during the study. All authors have agreed to publish this manuscript.
Availability of Data and Material
All data generated or analyzed during the study are included in this published article.

Code Availability
Not applicable.

ORCID
Badr Alzahrani https://orcid.org/0000-0003-4158-640X
Mohamed M. S. Gaballa https://orcid.org/0000-0003-2949-430X
Ahmed A. Tantawy https://orcid.org/0000-0002-5017-686X
Maha A. Moussa https://orcid.org/0000-0002-6987-9012
Salma A. Shoulah https://orcid.org/0000-0003-2115-1572
Said M. Elshafae https://orcid.org/0000-0001-6285-5483

Author Contributions
Conceptualization: BA, AAT, MMSG, SME. Data curation: AAT, MMSG, SME. Formal analysis: BA, AAT, MMSG, SME. Funding acquisition: BA, AAT. Investigation: AAT, MMSG, SME. Methodology: AAT, MMSG, SME. Supervision: AAT, SME. Validation: BA, AAT, MMSG, SME, MAM, SAS. Writing—original draft: BA, MMSG. Writing—review & editing: AAT, SME. Approval of final manuscript: all authors.

Conflicts of Interest
The authors declare that they have no potential conflicts of interest.

Funding Statement
No funding to declare.

References

An unusual case of microsatellite instability–high/deficient mismatch repair (MSI-H/dMMR) diffuse large B-cell lymphoma revealed by targeted gene sequencing

Bogyeong Han¹, Sehui Kim¹, Jiwon Koh¹, Jeong Mo Bae¹, Hongseok Yun², Yoon Kyung Jeon¹,³

¹Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul; ²Center for Precision Medicine, Seoul National University Hospital, Seoul; ³Cancer Research Institute, Seoul National University, Seoul, Korea

Microsatellite instability-high/deficient mismatch repair (MSI-H/dMMR) status has been approved as a tissue-agnostic biomarker for immune checkpoint inhibitor therapy in patients with solid tumors. We report the case of an MSI-H/dMMR diffuse large B-cell lymphoma (DLBCL) identified by targeted gene sequencing (TGS). A 90-year-old female who presented with vaginal bleeding and a large mass in the upper vagina was diagnosed with germinal center-B-cell-like DLBCL, which recurred at the uterine cervix 9 months after chemotherapy. Based on TGS of 121 lymphoma-related genes and the LymphGen algorithm, the tumor was classified genetically as DLBCL of EZB subtype. Mutations in multiple genes, including frequent frameshift mutations, were detected by TGS and further suggested MSI. The MSI-H/dMMR and loss of MLH1 and PMS2 expression were determined in MSI-fragment analysis, MSI real-time polymerase chain reaction, and immunohistochemical tests. This case demonstrates the potential diagnostic and therapeutic utility of lymphoma panel sequencing for DLBCL with MSI-H/dMMR.

Key Words: Diffuse large B-cell lymphoma; Microsatellite instability; Deficient mismatch repair; Targeted gene sequencing

Received: April 22, 2021 Revised: October 14, 2021 Accepted: October 15, 2021
Corresponding Author: Yoon Kyung Jeon, MD, PhD, Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea
Tel: +82-2-740-8323, Fax: +82-2-743-5530, E-mail: ykjeon@snu.ac.kr

Microsatellite instability (MSI) is a condition of genetic hypermutability caused by an impaired DNA mismatch repair system, which arises through a germline or somatic mutation, or a promoter hypermethylation [1]. MSI-high (MSI-H) or mismatch repair deficiency (dMMR) status in solid tumors predicts their response to immune checkpoint blockade [2]. Thus, in 2017, the U.S. Food and Drug Administration (FDA) approved the programmed death-1 (PD-1)–blocking agent pembrolizumab for treatment of unresectable or metastatic MSI-H/dMMR solid tumors, irrespective of cancer type; this was the first cancer site/histology-agnostic biomarker approved by the FDA [3]. However, the prevalence of MSI is highly variable across cancer types; it is very low (< 1%) in non-Hodgkin lymphomas, except immuno-deficiency-related lymphomas [4-8]. MSI has been studied widely in colon cancer and endometrial cancer but only rarely in diffuse large B-cell lymphoma (DLBCL) [4,8,9]. The clinical relevance of MSI in patients with DLBCL, in terms of prognostic significance or as a predictor of the response to immune checkpoint blockade, is unknown. Here, we report an unusual case of MSI-H/dMMR DLBCL initially identified by clinical targeted gene sequencing (TGS) and subsequently confirmed by MSI testing.

CASE REPORT

A 90-year-old female presented with vaginal bleeding. A protruding polypoid mass measuring 5.8 cm was detected in the upper vagina by pelvic magnetic resonance imaging. Positron emission tomography revealed an additional focal hypermetabolic lesion in the presacral area. A tissue biopsy was conducted under the suspicion of cervical cancer. The patient was diagnosed with DLBCL with a germinal center B-cell-like (GCB) phenotype determined by immunohistochemistry (IHC)-based Hans algo-
rithm, clinical stage of IIA, and Eastern Cooperative Oncology Group performance status of 1. Rituximab and reduced-dose CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) resulted in complete remission (CR), but after 9 months, she suffered local relapse at the uterine cervix and underwent tumor excision. Microscopically, the tumor was composed of atypical lymphoid cells with a centroblastic morphology and diffuse arrangement. IHC for CD3, CD20, C-MYC, BCL-2, BCL-6, CD10, MUM1, Ki-67, CD8, and programmed death-ligand 1 (PD-L1) (clone E1L3N) and in situ hybridization for Epstein-Barr virus (EBV) and fluorescence in situ hybridization (FISH) for MYC were performed. Tumor cells were positive for CD20, CD10, and BCL-6 but negative for MUM1, BCL-2, C-MYC, and EBV. The Ki-67 labeling index of tumor cells was 80% (Fig. 1A–H). MYC translocation was not observed in FISH (data not shown). The tumor was diagnosed again as DLBCL with a GCB phenotype. Scattered CD3+ or CD8+ tumor-infiltrating lymphocytes were observed (Fig. 1I, J), and PD-L1 was expressed in tumor-associated macrophages and in about 5% of tumor cells (Fig. 1K). TGS was performed using a customized panel comprising 121 lymphoma-related genes and formalin-fixed paraffin-embedded sections of the surgical specimen; it revealed 16 mutations across 15 genes including ARID1A, DNMT3A, PDCD1, SETD2, PDGFRA, TET2, PRDM1, CARD11, ATM, KMT2D, B2M, CREBBP, CIITA, GNA13, and BTK (Table 1). The tumor was genetically classified as EZB subtype DLBCL according to the LymphGen algorithm (https://llmpp.nih.gov/lymphgen/index.php) [10]. In addition, 34 further mutations (27 missense mutations, 5 frameshift mutations, 1 inframe indel mutation, and 1 stop-gain mutation) in 28 genes were identified as rare germline variants or variants of uncertain significance (data not shown). Although microsatellite markers were not included in this lymphoma panel, the unusually high number of variants, including a large number of frameshift mutations, suggested MSI. A fragmentation assay based on the Bethesda guidelines, immunohistochemistry of four proteins involved in the mismatch repair (MMR) pathway (MLH1, MSH2, MSH6 and PMS2), and a U-TOP MSI detection test (Seasun Biomaterials Inc., Daejeon, Korea) were conducted. On the fragmentation assay, four (BAT25, BAT26, D2S123, and D17S2720) of the five Bethesda microsatellite markers showed features suggestive of MSI (data not shown). A loss of MLH1 and PMS2

![Fig. 1. Microscopy and immunohistochemical findings.](https://jpatholtm.org/https://doi.org/10.4132/jptm.2021.10.15)
protein expression was detected by immunohistochemistry (Fig. 1L–O), and the U-TOP MSI detection test, which uses five quasi-monomorphic mononucleotide markers that do not require samples of normal tissue from the patient for comparison [11], showed MSI in all five markers, confirming the MSI-H/dMMR status of the tumor (Fig. 2). The patient was treated by radiotherapy of the involved site and has been in metabolic CR for the past 7 months.

**DISCUSSION**

MSI-H or dMMR status is the first tissue-agnostic biomarker approved by the FDA for cancer therapy and, more specifically, for pembrolizumab therapy [3]. Patients with MSI-H/dMMR tumors have shown a favorable clinical response to PD-1 blockade, regardless of cancer type [2]. This illustrates the need for MSI-H tumor detection. MSI-PCR of the Bethesda panel to assess three dinucleotide repeats (D2S123, D5S346, D17S250) and two mononucleotide repeats (BAT26, BAT25) is the gold standard for detecting MSI. According to the revised Bethesda guidelines, pentaplex PCR with five quasi-monomorphic mononucleotide repeats can detect MSI with high sensitivity and specificity and might not need matched normal tissue [1]. Together with immunohistochemistry for MMR proteins, it allows determination of MMR status [1]. MSI testing performed in endometrial, colorectal, and gastric cancers has revealed high variability in the frequency of MSI-H among cancers [4,12]. However, the frequency of MSI-H non-Hodgkin lymphoma generally is low (0%–2%), which has hampered standardized MSI testing as a routine diagnostic method [4,12]. Recently, next-generation sequencing (NGS)-based TGS using a panel of tumor markers has been introduced for molecular pathologic diagnosis. In solid tumors, NGS-based MSI tests have shown high sensitivity and specificity in the absence of control normal tissue [4-7].

**Table 1. Variants found in DLBCL by targeted sequencing based on 121 lymphoma-related genes (excluding rare germline variants or variants of uncertain significance)**

<table>
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<th>Gene</th>
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<th>AA change</th>
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<td>NM_000061.2</td>
<td>12</td>
<td>c.993A&gt;G</td>
<td>p.Ile331Met</td>
<td>45.38</td>
</tr>
</tbody>
</table>

Chr, chromosome; AA, amino acid; VAF, variant allelic frequency.

**Fig. 2.** Microsatellite instability (MSI) test results. MSI was detected in all five quasi-monomorphic markers using the U-TOP MSI detection test, revealing the MSI-high (MSI-H) status of the tumor. Genomic DNA from HeLa cells was used as a negative control.
Although unified criteria for detection of MSI by NGS are lacking, many laboratories are using MMR-related genes and MSI markers, as well as bioinformatics algorithms, for tumor diagnosis [4-7].

The FDA has approved PD-1 blockade therapy based on its efficacy in patients with hematologic malignancies, including Hodgkin lymphoma and primary mediastinal large B-cell lymphoma [13,14]. A recent study demonstrated the potential benefit of PD-1 blockade in combination with R-CHOP in patients with treatment-naive DLBCL overexpressing PD-L1 [15]. However, both the MSI landscape and efficacy of PD-1 blockade for MSI-H lymphoma remain unclear. A previous study reported DLBCL with defects in DNA repair genes, including tumor suppressor genes, MMR-related genes (e.g., MSH2 and MSH6), and non-homologous end-joining pathway-related genes, and it suggested an association of genomic instability phenotype with tumorigenesis of DLBCL [16]. However, recent analyses of NGS data showed that MSI-H in non-Hodgkin lymphoma, including DLBCL, is either extremely rare (<1%) or not a feature of these tumors [4-7]. In contrast to those reports, a study using a commercial MSI kit, based on eight mononucleotide repeat markers and two pentanucleotide repeat markers, identified MSI-H and microsatellite instability–low (MSI-L) in 3% (3/92) and 10% (9/92) of DLBCLs, respectively [9]. According to the authors, patients with MSI-H DLBCL tended to have a better prognosis than those with microsatellite stable DLBCL, although the difference was not significant [9]. They also reported that MSI-L DLBCL was associated with a poor response to chemotherapy [9]. These results suggest that MSI status could be a useful biomarker for DLBCL.

Reports on the prognostic and therapeutic impacts of molecular genetic classification of DLBCL indicate the potential utility of TGS in individually tailored treatment for DLBCL [10,17]. The LymphGen algorithm classified DLBCLs into five genetic subtypes: MCD (including MYD88 L265P and CD79B mutations), BN2 (including BCL6 translocations and NOTCH2 mutations), N1 (including NOTCH1 mutations), EZB (including EZH2 mutations and BCL2 translocations), and A53 (aneuploid with TP53 inactivation) [10]. Our inclusion of 121 lymphoma-related genes allowed successful genetic determination of the tumor as EZB, which is the most common genetic subtype of GCB-DLBCL [10]. TGS also revealed frequent frameshift mutations and C:G → A:T transversions, as previously reported in MMR-mutated DLBCLs [16]. Although MMR gene mutations could not be identified directly using our lymphoma panel, the unique mutational pattern of our patient’s tumor was suggestive of an MSI-H/dMMR DLBCL. The MSI status of the tumor was confirmed by two kinds of MSI tests for microsatellite markers (i.e., MSI-fragmentation assay and MSI-pentaplex real-time PCR using U-TOP MSI detection test), as well as by immunohistochemistry for four MMR proteins.

However, because our patient did not receive PD-1 blockade therapy, the efficacy for treating this type of tumor could not be determined. Nonetheless, this case shows that MSI-H/dMMR can be present in DLBCL, albeit rarely, and the utility of TGS for detection of MSI-H/dMMR in hematolymphoid malignancies.

**Ethics Statement**

This study was approved by the Institutional Review Board (IRB) of Seoul National University Hospital (No. 2012-160-1184) and written informed consent from the patient was waived by IRB decision.

**Availability of Data and Material**

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

**Code Availability**

Not applicable.

**ORCID**

Bogyeong Han https://orcid.org/0000-0003-0391-7415
Selhui Kim https://orcid.org/0000-0002-6640-3051
Jiwon Koh https://orcid.org/0000-0002-7687-6477
Jeong Mo Bae https://orcid.org/0000-0003-0462-3072
Hongseok Yun https://orcid.org/0000-0003-2776-5954
Yoon Kyung Jeon https://orcid.org/0000-0001-8466-9681

**Author Contributions**

Conceptualization: YKJ, JMB. Data curation: BH, SK. Formal analysis: JK, JMB, HY. Investigation: BH, JMB, HY. Visualization: BH, SK. Writing—original draft: BH, SK. Writing—review & editing: JK, YKJ. Approval of final manuscript: all authors.

**Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

**Funding Statement**

This study was funded by Basic Science Research Program (grant No.: NRF-2016R1D1A1B01015964) through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology (MEST).

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Colorectal adenocarcinoma with enteroblastic differentiation (CAED) is a rare subtype of colorectal adenocarcinoma characterized by increased α-fetoprotein (AFP) production and the expression of at least one enteroblastic marker including AFP, glypican 3 (GPC3), or Spalt like transcription factor 4 (SALL4). We report a case of a 26-year-old female who presented with low back pain and constipation which persisted despite supportive measures. Imaging revealed multiple liver lesions and enlarged retroperitoneal nodes. Tumor markers including AFP were markedly elevated. On biopsy, samples from the liver revealed infiltrating glands lined by columnar-type epithelium with mostly eosinophilic granular to focally clear cytoplasm. By immunohistochemistry, the tumor showed immunoreactivity with AFP, hepatocyte antigen, GPC3, SALL4, CDX2, SATB2, and cytokeratin 20. A colonoscopy performed subsequently revealed a mass in the sigmoid colon and biopsy of this mass revealed a similar histology as that seen in the liver. A diagnosis of CAED was made, following the results of gene expression profiling by the tumor with next-generation sequencing which identified pathogenic variants in MUTCYH, TP53, and KDM6A genes and therefore supported its colonic origin. Cases such as this underscores the use of ancillary diagnostic techniques in arriving at the correct diagnosis in lesions with overlapping clinicopathologic characteristics.

**Key Words:** Colorectal neoplasms; Colorectal adenocarcinoma with enteroblastic differentiation; Alpha fetoprotein

Colorectal adenocarcinoma with enteroblastic differentiation is a rare subtype of colorectal adenocarcinoma characterized by expression of enteroblastic markers including glypican 3 (GPC3), Spalt like transcription factor 4 (SALL4), and α-fetoprotein (AFP) [1]. The incidence of CAED compared with other types of colorectal carcinoma is extremely rare, possibly because the colorectum originates from the hindgut endoderm [4,5].

AFP is an oncofetal glycoprotein produced by the fetal liver, yolk sac, and a small amount from the fetal gastrointestinal epithelium [6]. Serum levels are elevated after birth but decrease significantly by the second year of life [4]. Thus, an increase in serum AFP is abnormal in adults as it is produced by certain tumors, making it a useful tumor marker for the diagnosis and monitoring of treatment [7].

In addition to AFP, the oncofetal proteins GPC3 and SALL4, expressed in germ cell tumors, are immunohistochemically associated with AFP-producing gastric cancers and their related counterparts. Other markers include cytokeratin (CK) 7, CK 20, and caudal-type homebox 2 (CDX2) which aid in differentiating between tumors of the breast, gynecological tract, liver, and lung origin [8].
Here we present a case of chemo-refractory CAED in a young female who initially presented with multiple liver lesions, and a mass in the sigmoid colon, with diagnostic considerations of a primary hepatocellular carcinoma (HCC), germ cell tumor, and CAED, and the use of ancillary diagnostic techniques in arriving at the correct diagnosis.

**CASE REPORT**

This is a case of a 26-year-old female who presented clinically with a history of low backache and constipation, which persisted despite supportive measures. Imaging studies revealed multiple liver lesions, with enlarged retroperitoneal lymph nodes. Tumor markers at presentation included an AFP of 39,493 ng/mL, carcinoembryonic antigen of 3,040 ng/mL, cancer antigen (CA) 19-9 of 621 U/mL, and CA 125 of 42 U/mL.

A biopsy of her liver nodules was performed and histopathologic review of tissue sections revealed segmental necrosis with infiltrating adenocarcinoma (Fig. 1). The tumor cells had moderate amounts of mostly granular eosinophilic to focally clear cytoplasm (Fig. 2A) and nuclei with densely homogenous chromatin and occasional inconspicuous nucleoli (Fig. 2B). Immunohistochemistry (IHC) revealed that the tumor cells were immunoreactive for AFP (Fig. 3A), Sal-like protein 4 (Fig. 3B), CK20 (Fig. 3C), and showed strong diffuse immunoreactivity with CDX2 (Fig. 3D). Hepatocyte specific antigen was also positive on IHC. However, CK7, PAX8, and S100 were all negative. A colonoscopy performed subsequently, revealed a mass in the sigmoid colon and biopsy of this mass revealed a similar histology as that seen in the liver (Fig. 4A–C). IHC expression in the colonic tumor showed similar expression as that in the liver. Considering the fact that CDX2 positivity would be unlikely in a primary HCC, a diagnosis of CAED was favored based on a combination of serum tumor markers, tumor morphology, and IHC expression in the tumor cells. Additional testing performed on the tumor cells include human epidermal growth factor receptor 2, which was interpreted as equivocal (2+) on IHC and negative by fluorescence in situ hybridization. KRAS mutations were identified in the tumor cells, but programmed death-ligand 1 (PD-L1) was negative. IHC stains for mismatch repair (MMR) proteins MLH1, MSH2, MSH6, and PMS2 showed no loss of expression (no evidence of MMR deficiency). She was commenced on targeted therapy including FOLFOX (leucovorin calcium [folinic...
acid], fluorouracil, and oxaliplatin) and bevacizumab. However, with suboptimal improvement in her clinical disease, a second opinion was sought.

She underwent additional imaging studies which revealed new satellite lesions in the liver and previously undiscernible lesions in bilateral ovaries, which was highly suspicious for advancing metastatic disease. With the discovery of new lesions in the ovary, her previous biopsies from the liver nodules and colonic mass were re-reviewed and a diagnosis of poorly differentiated carcinoma, most suggestive of yolk sac tumor was rendered in the original

**Fig. 3.** Immunohistochemical stains show that the tumor cells are immunoreactive for α-fetoprotein (A), Sal-like protein 4 (B), cytokeratin 20 (C), and CDX2 (D).

**Fig. 4.** (A) Biopsy of sigmoid colon mass showing infiltrative malignant glands. (B) Higher magnification of colonic mass showing cells with moderate amounts of mostly granular eosinophilic to focally clear cytoplasm (arrows). (C) Higher magnification of colonic mass showing tumor cells with moderate amounts of mostly granular eosinophilic cytoplasm and a cell with inconspicuous nucleolus (arrow).
liver and colonic tumor specimens. Other differentials that were considered included a mixed germ cell tumor or teratoma. She was commenced on chemotherapy for germ cell tumors (cisplatin, etoposide, and bleomycin) but showed disease progression on imaging studies. This prompted a decision to send the specimens from the liver and colon for tumor gene expression profiling by next-generation sequencing (NGS). NGS identified several pathogenic variants including MUTYH (exon 13, p.G396D), TP53 (exon 5, p.R175H), and KDM6A (exon 16, p.S552fs). Additionally, estrogen receptor and progesterone receptor by IHC were both negative. Based on the constellation of clinical findings and test results, a diagnosis of CAED was upheld and she is being evaluated for autologous transplant for chemo-refractory CAED.

**DISCUSSION**

The incidence of AFP-producing gastric carcinoma has been documented to be within the range of 1.3%–15% worldwide with the incidence of the colorectal variant being significantly lower compared to the gastric [9]. This was first reported by Nakajima et al. in 1985 [10]. Prior reports suggest that CAED is more common in males and tends to be located in the left colon [1,2].

Furuya et al. in their case report in 2011 reported the first case of primary AFP-producing clear cell adenocarcinoma of the colon in an 81-year-old man, which is considered extremely rare when compared to that of gastric origin [11]. They made a distinction between a primary colonic tumor and metastasis from the gynecological tract or other organs by whole-body computed tomography (CT) and immunostaining. CT results revealed tumor deposits in the colon and lung only with CK7 negative and CD20 positive immunoreactivity [11].

Histologically, the tumor cells display a spectrum of morphologic patterns. One report describes tumor cells that displayed typical adenocarcinoma morphologies composed of cuboidal or columnar cells with clear cytoplasm resembling the primitive gut [12]. The enteroblastic component was seen occurring concurrently with typical well or moderately differentiated tubular adenocarcinomas [12]. Another report describes the tumor’s morphology as characterized by a solid-type poorly differentiated carcinoma, mainly composed of eosinophilic cells with focal glandular differentiation on hematoxylin and eosin stains [13].

Our patient is a young, 26-year-old female, an interesting demographic in contrast to most documented cases of CAED. Her primary tumor was located on the left side (sigmoid colon) with multiple liver, nodal, and bilateral ovarian involvements warranting the diagnostic consideration of mixed germ cell tumor especially with unresponsiveness to conventional chemotherapy. However, with tumor progression on imaging following the administration of chemotherapy for germ cell tumor and the IHC profile, a diagnosis of chemo-refractory CAED was ultimately favored, based on the constellation of clinicopathologic findings and results of gene expression of the tumor cells by NGS. Of note is that the MUTYH gene encodes a DNA glycosylase involved in oxidative DNA damage repair and mutations in the gene predispose to colon and stomach cancers. A germline mutation in TP53 predisposes to multiple cancers, including colorectal cancer.

Clear cell changes were proposed by Eloy et al. [14] to be a result of degenerative changes with an accumulation of cytoplasmic lipid-like material and not necessarily the accumulation of cytoplasmic glycogen and mucin. Therefore, mucin and glycogen may go undetected in some cases of CAED [14]. In our case, we observed mostly eosinophilic granular cytoplasm, with only a focal clear cell component (Fig. 2).

AFP production in gastric carcinoma was suggested by Matsunou et al. [15] to be due to a form of retro-differentiation towards fetal intestine as clear cell carcinoma with morphology similar to the developing gut epithelium, or fetal hepatocytes, i.e., hepatoid carcinoma. AFP-producing colorectal cancers are generally observed to be poorly to moderately differentiated, a histological characterization that makes it different from AFP-producing gastric cancers which are commonly poorly differentiated. However, both rapidly progresses and metastasizes frequently to the liver with a poor prognosis [4]. Poor differentiation, deep submucosal invasion, lymphatic invasion, vascular invasion, or a positive resection margin are risk factors for lymph node metastasis in submucosal colorectal cancers [16].

Due to limited data on CAED, only very few studies and case reports have documented the results of ancillary studies including genomic [1,13] and microsatellite instability (MSI) characteristics [1]. One study reports an MSI-high rate in CAED of 12.2% [1], which is almost similar to that reported in conventional CRC [17]. The tumor in this case showed intact expression of MMR proteins (MLH-1, PMS-2, MSH-2, MSH-6) by IHC in CAED. NGS analyses from one study revealed that the most frequently mutated gene in CAED is TP53, at a rate of 52.4% [1] which is almost similar to the TP53 mutation rate in conventional CRC [18,19]. The KRAS mutation from the same study was reported at 27.6%, similar to the rate in conventional CRC [20]. We detected a KRAS mutation in our CAED case; however, PD-L1 was negative. Other mutated genes that have been reported in CAED

https://jpatholtm.org/
include \( APC \) (44.8\%), \( BRAF \) (20.7\%), \( NRAS \) (17.2\%), and \( PIK3CA \) (17.2\%) [1]. We recognize the limitation of this case as diagnosis relied on biopsy specimens from the colonic and liver tumors. However, given the advanced stage at disease presentation, neoadjuvant treatment modalities were pursued for possible disease control before surgery.

There are no specific treatment guidelines yet for patients with AFP-producing colorectal cancers. Therefore, treatment regimens are employed according to multiple guidelines and personal experience. Most will undergo the conventional treatments for colorectal cancers which include surgery and chemotherapy with many dying within a year of initiating therapy [21]. Our patient is being evaluated for autologous stem cell transplant following disease progression on standard chemotherapy for conventional CRC and germ cell tumor.

In summary, CAED is a rare subtype of CRC with poor prognosis and poses diagnostic difficulties on account of its clinical and histopathologic similarities to primary HCC and germ cell tumors. Ancillary diagnostic modalities including gene expression profiling in the tumor cells by DNA sequencing techniques, may be necessary to correctly diagnose this entity, especially in challenging cases as was encountered in this case. Therefore, pathologists need to be aware of this rare tumor, as a missed diagnosis could significantly impact the clinical care and outcome of patients.

**Ethics Statement**

As this is a case study without identifiers, our institution does not require approval from the institutional review board or its equivalent.

**Availability of Data and Material**

Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

**Code Availability**

Not applicable.

**ORCID**

Evi Abada https://orcid.org/0000-0001-5061-2687

Ifeoma C. Anaya https://orcid.org/0000-0001-9941-6660

Anthony Lebbos https://orcid.org/0000-0003-4048-4217

**Author Contributions**

Conceptualization: EA, RB. Data curation: EA, RB. Methodology: all authors. Project administration: EA. Resources: all authors. Supervision: all authors. Visualization: all authors. Writing—original draft: all authors. Writing—review & editing: all authors. Approval of final manuscript: all authors.

**Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

**Funding Statement**

No funding to declare.

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Recurrence malignant solitary fibrous tumor of the scalp: a case report and literature review

Ahmed Rabie1, Abdulkarim Hasan2, Yasein Mohammed1, Ayman Abdelmaksoud1, Ali A. Rabaan4

1Department of Pathology, Faculty of Medicine, Al-Azhar University, Damietta Branch, Damietta;
2Department of Pathology, Faculty of Medicine, Al-Azhar University, Cairo;
3Mansoura Dermatology, Venereology and Leprosy Hospital, Mansoura, Egypt;
4Molecular Diagnostic Laboratory, Johns Hopkins Aramco Healthcare Dhahran, Dhahran, Saudi Arabia

Solitary fibrous tumor (SFT) is a rare type of fibroblastic mesenchymal neoplasm that first was discovered in the pleura but can also affect the peritoneum, lungs, mediastinum, and skin. Cutaneous malignant SFT is an extremely rare tumor that resembles dermatofibrosarcoma protuberance (DFSP) histologically and immunohistochemically. Herein, we describe a case of malignant SFT that presented as a recurrent mass on the scalp. The first lesion was totally excised one year before recurrence and was diagnosed as a DFSP based on the histopathology and cluster of differentiation 34 immunostaining positivity. Re-examination of the previously examined specimen was considered. Activator of transcription 6 positivity was also detected in the tissue, confirming the diagnosis of a recurrent malignant SFT rather than DFSP. There was no evidence of recurrence, locoregional, or distant metastases at six months after lesion removal with a safety margin.

Key Words: Solitary fibrous tumor; Skin cancer; Immunohistochemistry; STAT6

CASE REPORT

A 51-year-old male patient who was seemingly healthy presented with a recurrent subcutaneous occipital mass one year after excision. The mass was diagnosed as dermatofibrosarcoma protuberance (DFSP) based on histomorphology and CD34-positive immunostaining, with a size of 5×4 cm and free margins of at least 0.2 cm. The clinical examination of the new lesion revealed an 8×7 cm subcutaneous mass with overlying skin ulceration, raising the possibility of recurrent DFSP or a neoplastic epithelial lesion consistent with squamous cell carcinoma. The radiological assessment showed no associated lytic or sclerotic bony lesions. For histopathological examination, the lesion was removed surgically with a safety margin. Gross investigation revealed ulcerated skin overlying a well-defined, lobulated, white, and focally...
necrotic mass (Fig. 1). A spindle cell tumor organized in irregular short bundles with staghorn blood vessels was revealed under microscopic analysis. Tumor cells had large, rounded vesicular nuclei and conspicuous micronuclei, with a small to moderate proportion of eosinophilic cytoplasm. Tumor cell nuclei exhibited a moderate degree of pleomorphism and frequent mitosis, approximately seven mitoses per 10 high-power fields (HPFs), with occasional atypical forms and foci of necrosis (Fig. 2). The tumor had invaded the overlying skin. A storiform pattern was not present. There were no dedifferentiated or anaplastic spots. All surgical margins were devoid of tumor cells, with the nearest free margin measuring 0.5 cm (side and deep margins).

In the first immunohistochemical panel study, CD34 was significantly positive in most tumor cells, but SMA, desmin, S100, pan-CK, and epithelial membrane antigen showed negative results. B-cell lymphoma 2 (BCL2) marker exhibited weak focal staining. Based on the histomorphological and immunohistochemical results, histopathological differential diagnosis was performed, including malignant solitary fibrous tumor, fibrosarcoma on top of dermatofibrosarcoma, and malignant peripheral nerve sheath tumor. Further immunohistochemical investigation of SOX10, STAT6, and Ki67 was considered, where the Ki67 labeling index was 30%, and STAT6 exhibited a strong diffuse nuclear positivity (Fig. 3), whereas SOX10 was not expressed. Re-cut and re-examined tissue blocks and slides from previous surgery confirmed the same histomorphology, including the lack of storiform pattern. STAT6 immunohistochemical staining of the primary lesion showed diffuse nuclear positivity.

Consequently, the final diagnosis was recurrent malignant SFT (TNM stage T3). Metastatic risk assessment revealed an interme-

Fig. 1. A gross picture showing ulcerated skin (A) and fairly defined lobulated whitish mass with central focal necrosis (B).

Fig. 2. A histopathology picture showing a cellular spindle cell tumor arranged in irregular short bundles with staghorn blood vessels (A) and mitotic figures (circles) (B).
diate-risk solitary fibrous tumor. The patient was referred to the oncology department for further evaluation and showed no signs of metastasis at first presentation. Six months later, regular follow-up indicated no evidence of recurrence or distant metastasis.

DISCUSSION

SFT first was described in 1931 by Klemperer and Rabin as “localized fibrous mesothelioma” [10]. SFT and hemangiopericytoma previously were considered to be distinct entities; however, beginning with the 2013 World Health Organization classification of soft tissue tumors, SFT and hemangiopericytoma are now considered the same neoplasm—except in the central nervous system, where meningeal hemangiopericytoma remains a distinct entity [4]. SFT is frequently found in adults aged 20 to 70 years but has been recorded in children on rare occasions [11]. Although SFTs were initially classified as pleural tumors, they can be found in various tissues, such as the liver, lungs, kidneys, thyroid, neurological system, soft tissue, and skin [9,12]. Cutaneous SFT is an extremely rare neoplasm, presenting as a painless, superficial, and well-circumscribed mass that can be confused clinically with lipoma or epidermal cyst [3]. A summary of previously reported cases of scalp SFT and the current one is illustrated in Table 1 [13-24]. Confirming a diagnosis of unusual skin and soft tissue mass lesions, including SFT, is challenging and requires careful sampling of the tumor mass followed by careful pathological examination [25,26]. Although fine-needle aspiration cytology is used frequently to diagnose skin and soft tissue masses [27], no definitive cytological findings of SFT have been identified. SFT requires suitable clinical and radiological correlation and immunohistochemical tests for preoperative diagnosis [28,29]. SFT is classified histologically as storiform, hemangiopericytic, herring-bone, diffuse sclerosing, or neural-type palisading. SFT spindle cells proliferate in a “patternless” manner and form dense collagen bundles embedded in the stroma with elaborate vasculature [3]. Malignant SFTs are differentiated from benign SFTs by their high degree of cellularity, size greater than 5 cm, number of mitoses per HPF, presence of immature or pleomorphic tumor cells, and necrosis foci.

A correlation between number of mitoses per HPF and prognosis was observed. At least four mitoses per 10 HPFs are associated with metastasis and recurrence [4,30]. There are differential diagnoses for malignant SFT, such as benign SFT, DFSP, nerve sheath tumors, synovial sarcoma, liposarcoma, and leiomyosarcomas [31]. Clinico pathological correlation of SFT might not be sufficient to reach a definitive diagnosis. SFTs previously were diagnosed by immunohistochemical expression of various markers, including CD34, CD99, and BCL2; however, these markers carry a poor specificity [7,32]. CD34 is expressed highly in SFTs but also in other tumors that are included in the differential diagnosis of SFTs—namely, DFSP. CD34 is absent in around 5% to 10% of SFTs, mostly in dedifferentiated and ma-
lignant instances [33]. Cancers of the nervous system, melanocytic cells, fat, and smooth muscle can be recognized by their positivity for tumor-specific markers such as S100, desmin, and SMA, which are non-reactive with SFT tumor cells [8]. Our case was misdiagnosed previously as DFSP based on histomorphology and CD34 positivity. However, the absence of a storiform pattern in the tissue and the presence of the STAT6 marker validated our findings on both initial and recent/recurrent specimens. Detection of the NAB2:STAT6 fusion gene in SFTs is a reliable distinction from other spindle cell tumors.

NAB2 is a transcriptional repressor of the early growth response transcription factor, and its fusion with STAT6 can convert the repressor into a transcriptional activator, driving neoplastic progression [34]. This finding led to identification of STAT6 immunohistochemical study as a highly sensitive and specific marker for SFTs, whereas DFSP is STAT6-negative [35]. Surgical resection with safety margins is the preferred treatment for both malignant SFTs and DFSP, but chemo-and radiotherapy can be challenging. Adjuvant chemotherapy and radiotherapy are not used widely due to the rarity of SFTs. However, chemotherapy can be beneficial in malignant situations where the tumor appears to be radiation-responsive, implying a more significant chance of reduction in local recurrence [36,37].

While SFT of the scalp is uncommon, malignant SFT is even more uncommon and should be distinguished carefully from other spindle cell tumors of the skin, especially DFSP. To the best of our knowledge, this case is the third report of malignant SFT on the scalp and the first reported case of recurrent SFT at this anatomical site. CD34 immunostaining is insufficient to specify a malignant SFT of the skin. Thus, STAT6 markers should be included in the immunohistochemical panel for differential diagnosis of challenging cases of superficial spindle cell neoplasms.

**Ethics Statement**

Ethical committee approval from the Faculty of Medicine, Al-Azhar University board is provided (ID: IRB 00012367-21-03-008). Informed consent was obtained from the patient.

**Availability of Data and Material**

All data generated or analyzed during the study are included in this published article (and its supplementary information files).

**Code Availability**

Not applicable.
ORCID
Ahmed Rabie https://orcid.org/0000-0002-4033-8554
Abdulkarim Hasan https://orcid.org/0000-0003-0991-306X
Yasein Mohammed https://orcid.org/0000-0002-1290-5910
Ayman Abdelmaksoud https://orcid.org/0000-0003-4848-959X
Ali A. Rabaan https://orcid.org/0000-0002-6774-9847

Author Contributions

Conflicts of Interest
The authors declare that they have no potential conflicts of interest.

Funding Statement
No funding to declare.

References
The adenoma-carcinoma sequence of carcinogenesis in the colorectum, featuring the progressive accumulation of alterations of APC, KRAS, p53, and other genes, was elucidated in the 1990s and has since become a textbook example of the stepwise model of carcinogenesis. In recent decades, our understanding of colorectal dysplasia has expanded through the study of the clinical, morphologic, molecular aspects of serrated dysplasia and non-conventional dysplasia in inflammatory bowel disease (IBD). Hence, we read with interest Choi’s timely review of the seven morphologic subtypes of non-conventional dysplasia in IBD [1].

The assessment of dysplasia in the context of IBD is a widely accepted and long-held area of diagnostic difficulty, associated with fair to moderate interobserver agreement even among subspecialty pathologists [2]. The question that naturally arises from the discovery of these unusual morphologic subtypes of dysplasia, that was to some extent based on retrospective study and generous resection specimens, is its applicability and impact on routine reporting of limited endoscopic biopsies. It is plausible that awareness of these morphologic subtypes could facilitate more accurate histologic recognition of dysplasia.

Alternatively, the devil’s advocate may posit that the subtlety of some of these morphologic subtypes may make the diagnosis of dysplasia in superficial biopsies inconclusive and ambiguous. Of note, hypermucinous dysplasia shows progressively less cytologic atypia towards the surface and is thus a more subtle form of dysplasia than we are accustomed to recognizing. Contrary to its low-grade appearance however, this subtype harbors a higher rate of KRAS alterations and aneuploidy [3,4], rendering it a high-risk lesion that should not be missed. From the clinical point of view, it would therefore be important to recognize non-conventional dysplasia, particularly the high-risk subtypes, and recommend short-term follow-up for these lesions, despite the concern about the ambiguous diagnosis for dysplasia. Conversely, from the healthcare systems point of view, a higher overall “indefinite for dysplasia” call rate may result in increased healthcare costs, particularly if the number needed to treat is high. Evidently, prospective evaluation of the impact on and of reporting, including inter-observer and intra-observer reproducibility among both subspecialty and general pathologists, is required, albeit conceivably limited by relatively small case numbers.

As with many areas in pathology, new knowledge often undergoes a process of “splitting,” investigation and then meaningful “lumping.” A prime example of this process, although out of the field of gastrointestinal pathology, is the study of subtypes of non-mucinous adenocarcinoma of the lung, that began with meticulous morphologic descriptions, such as lepidic, acinar, complex glandular, papillary, micropapillary and solid. Subsequent data on the prognostic implications of each of these subtypes facilitated meaningful grouping within a three-tier grading system formulated by the International Association for the Study of Lung Cancer, incorporated in the latest iteration of the World Health Organization classification of thoracic tumors. Similarly, further study of the behavior of the morphologic subtypes of non-conventional dysplasia in the colorectum may eventually allow formulation of broader categories in the future. In addition, one may contend that serrated lesions in IBD and sporadic serrated lesions could be united, given the similarity in clinical, pathologic, and molecular features.

As a matter of topical interest and therapeutic relevance, findings of a recent study by Kim et al. [5] concluded that NTRK-re-arranged colorectal carcinomas progress exclusively via the serrat-
ed pathway of neoplasia, thus expanding the molecular landscape of serrated colorectal lesions. To the best of our knowledge, oncogenic gene fusions including \textit{NTRK} fusions have yet to be studied in serrated lesions in IBD, and would indeed form an interesting research question.

In conclusion, while we have made strides in expanding the concepts of colorectal dysplasia, the present-day story of colorectal carcinogenesis in IBD is an unfinished one, with many curious questions, practical and scientific, yet to be solved.

\textbf{Ethics Statement}

Not applicable.

\textbf{Availability of Data and Material}

Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

\textbf{Code Availability}

Not applicable.

\textbf{ORCID}

Lavisha S. Punjabi https://orcid.org/0000-0003-0479-1690

\textbf{Author Contributions}

Conceptualization: LSP. Writing—original draft: LSP. Writing—review & editing: YNL, AT. Approval of final manuscript: all authors.

\textbf{Conflicts of Interest}

The authors declare that they have no potential conflicts of interest.

\textbf{Funding Statement}

No funding to declare.

\textbf{References}

Renal cell carcinoma concomitant with multiple myeloma

Anubhav Narwal¹, Prashant Ramteke¹, Lalit Kumar², Saumyaranjan Mallik¹

Departments of ¹Pathology and ²Medical Oncology, All India Institute of Medical Sciences, New Delhi, India

The concomitant presentation of multiple myeloma (MM) and renal cell carcinoma (RCC) in same patient is rare. The risk of developing a secondary neoplasia in primary malignancies is much higher (~31%) compared to the general population. The most common hematological malignancy associated with RCC is non-Hodgkin lymphoma. An association between MM and RCC has been reported previously, but literature on the topic is sparse. Secondary malignancies concomitant with MM are thought to be mostly due to the use of chemotherapeutic agents (alkylating agents) in addition to genetic and environmental factors. As treatment of RCC does not include alkylating agent, their role in post RCC, MM can be debated and indicates shared risk factors. These shared risk factors include age, lifestyle, environmental factors and genetic mutations [1-3]. Although many hypotheses have been proposed to establish an association between MM and RCC, none have been proven. Here we report an association of incidentally-detected clear cell RCC in a known case of MM.

Our patient was a 63-year-old male with a baseline diagnosis of MM IgA kappa (International Staging System II/Durie-Salmon Staging System III A) in 2011. He received four cycles of RD regimen (lenalidomide-dexamethasone) and exhibited a complete response. He underwent autologous stem cell transplant in 2011 followed by thalidomide maintenance. After treatment, the patient had his first relapse in November 2015, for which he received four cycles VRD (bortezomib-lenalidomide-dexamethasone), to which he exhibited a partial response and was kept on RD maintenance.

In January 2020, the patient was re-evaluated and was found to have 68% plasma cells on bone marrow. M-band was 5.5 g/dL; serum-free light-chain (SFLC) kappa 898 and lambda of 14.6 with SFLC ratio 61.5. Bone marrow fluorescent in-situ hybridization analysis demonstrated a deletion in the 16q23 chromosome. Whole-body positron emission tomography computed tomography scan showed multiple lytic lesions in the left parietal, sphenoid, basiocciput, clivus, bilateral mandible, humeri, sternum, ribs, femur, and sacrum along with a lower pole left renal mass. The patient was started on dexamethasone pulse therapy followed by bortezomib, pomalidomide and dexamethasone, meanwhile the renal mass was evaluated. Renal mass biopsy showed features of clear cell RCC. The patient has completed three cycles of the chemotherapy and is under follow-up monitoring (Fig. 1).

Ojha et al. [1] found that the relative risk of RCC is higher (89%) among post MM cases than MM in post RCC (51%) compared to the general population. The index patient is a 63-year-old male with MM and concomitant RCC as a second primary malignancy after a time gap of 120 months [1].

The recently proposed bidirectional association between MM and RCC, may be related to certain genetic risk factors. The roles of c-met receptor (family of MET gene) and its ligand hepatocyte growth factor are well-established in MM and RCC. Hence, MET may be a candidate gene for understanding the bidirectional association between MM and RCC [1,4]. Both synchronous and metachronous occurrence of MM and RCC have been described in literature, with time intervals between the two varying from 1 to 300 months for metachronous cases. There is a male predominance with median age of 50 years. The most common histological type was clear cell-type with occasional cases of chromophobe- and transitional-type RCC. RCC was treated by partial or complete nephrectomy in these cases. MM exhibited...
morphy ranging from classical to anaplastic variant [5,6].

However, considering the previous literature and the present case, myeloma should be suspected in RCC patients with new lytic bone lesions, and any renal masses should be carefully investigated in MM patients.

**Ethics Statement**

Not applicable.

**Availability of Data and Material**

Availability of Data and Material: Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

**Code Availability**

Not applicable.

**ORCID**

Anubhav Narwal https://orcid.org/0000-0003-0708-9346
Prashant Ramteke https://orcid.org/0000-0003-4163-2170
Lalit Kumar https://orcid.org/0000-0002-9210-2500
Saumyaranjan Mallick https://orcid.org/0000-0003-4366-5873

**Author Contributions**

Conceptualization: SM. Data curation: AN. Formal analysis: AN. Investigation: LK. Methodology: PR. Writing—original draft: AN, PR. Writing—review & editing: SM. Approval of final manuscript: all authors.

**Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

**Funding Statement**

No funding to declare.

**References**


Fig. 1. (A) Microphotograph of lytic lesion biopsy shows sheets of mature plasma cells with mild atypia. (B) Cells are immunopositive for CD138, (C) while negative for cytokeratin. (D) Biopsy of the renal mass shows cells arranged in sheets with clear cytoplasm and small nuclei. The cells are immunopositive for cytokeratin (E) and CD10 (F).
What’s new in molecular genetic pathology 2022: immune checkpoint inhibitor biomarkers and select solid tumors

Patricia C. Tsang, MD1 and Guoli Chen, MD2

1MedStar Washington Hospital Center, Pathology & Laboratory Medicine, Washington, DC, USA
2Geisinger Medical Laboratories, Geisinger Health, Danville, Pennsylvania, USA

Abstract

Predictive biomarker testing plays a critical role in targeted immuno-oncology, including the use of immune checkpoint inhibitors (ICI) for various solid tumors. Molecular advancements in cancers of the breast, kidney and brain have continued to propel tumor classification and precision therapy.

GENE FUSION NOMENCLATURE

The HUGO Gene Nomenclature Committee has released a new recommendation to denote gene fusion with a double colon, e.g., BCR::ABL [1].

IMMUNE CHECKPOINT INHIBITOR BIOMARKERS

- Programmed cell death ligand 1 (PDL1) immunohistochemistry (IHC) uses various scoring systems and cutoffs for particular antibody clones to quantify PDL1 expression as a predictive biomarker for specific FDA approved ICI. For example:

  - The PDL1 pharmDX 22C3 immunostain is a companion diagnostic for non small cell lung carcinoma (NSCLC) patients eligible for pembrolizumab immunotherapy.
  - The tumor proportion score (TPS) is calculated by dividing the total number of PDL1 positive tumor cells by the total number of tumor cells (≥ 1% positive cutoff).
  - The combined positive score (CPS) is based on dividing the number of PDL1 positive cells (tumor cells, lymphocytes, macrophages) by the number of tumor cells to predict pembrolizumab efficacy in metastatic gastric and gastroesophageal junctional carcinoma, bladder cancer and cervical cancer (Fig. 1). It has the same cutoff of ≥ 1%, using the 22C3 antibody clone.
  - The PDL1 SP142 clone is an FDA approved companion diagnostic for pembrolizumab with a cutoff of ≥ 10 mutations per megabase, regardless of solid tumor type.
  - The PDL1 SP142 clone is a companion diagnostic for atezolizumab.

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- High tumor mutational burden (TMB) may serve as a predictor of immunotherapy response. TMB reflects the number of somatic mutations per megabase of tumor genomic sequence. It can be determined by whole exome sequencing (WES) or targeted sequencing of gene panels (usually ≥ 200 genes). Cutoff values have not yet been unified and may vary in respect to different assay platforms and ICI drug targets. Recently, Foundation Medicine’s FoundationOne® CDx, which includes MSI and TMB assessment, was approved as a companion diagnostic for pembrolizumab with a cutoff of ≥ 10 mutations per megabase.

BREAST CARCINOMA

- NGS can simultaneously detect alterations of multiple genes involved in breast cancers. Clinically actionable genomic changes include ERBB2 (HER2) amplification (prevalence ~15%), BRCA1/BRCA2 mutations (5%–10%), PIK3CA mutations (30%–40%) and ETV6::NTRK3 fusion in secretory breast carcinoma (< 0.2% of breast cancers).
- BRCA1 and BRCA2 inactivating mutations carry a lifetime cumulative risk of 60% and...
55% to develop breast cancer, respectively. Poly-ADP-ribose polymerase (PARP) inhibitors are FDA approved targeted therapy for BRCA1/BRCA2 associated breast cancer.

- AP13K (phosphoinositide 3-kinase) inhibitor, alpelisib, is FDA approved for advanced breast cancers that are PIK3CA altered, HER2 (-) and hormone receptor (+).
- In a meta-analysis of 30% of breast cancers, TP53 mutation is associated with an increased likelihood of pathologic complete remission following chemotherapy, while wild type TP53 appears to induce cell cycle arrest instead of cell death, resulting in residual disease following chemotherapy [2].

- Multigenomic prognostic profiling is being used to predict the risk of breast cancer recurrence. Commercial platforms include:
  - OncoType DX® (Genomic Health Inc.): 21 gene reverse transcription PCR assay for predicting the likelihood of recurrence and chemotherapy benefit in hormone receptor (+) patients on endocrine therapy.
  - MammaPrint (Agendia, Inc.): 70 gene expression profile by microarray for predicting the risk of distant metastasis. Patients with high clinical risk but low genomic risk based on MammaPrint might not benefit from chemotherapy.
  - EndoPredict® (Myriad Genetics): 11 gene RNA expression profile for predicting the risk of recurrence in hormone receptor (+) / HER2 (-) patients who are node (+) or (-) on endocrine therapy.
  - Prosigna® (Veracyte): 50 gene reverse transcription PCR assay for assessing the risk of recurrence in 10 years for hormone receptor (+) early stage breast cancer and identifying the subset of patients who may not benefit from chemotherapy.

**RENAL CELL CARCINOMA (RCC)**

- In addition to recurrent VHL and 3p deletion, clear cell RCC frequently carries other genetic alterations. BAP1 or SETD2 mutated RCCs behave aggressively, while PBRM1 mutations predict a poor prognosis in localized RCC but a favorable outcome in advanced disease.
- Chromosome 7 or 17 polysomy and trisomy are the most common chromosomal changes in type 1 papillary RCC. Type 2 tumors are heterogeneous and may harbor other genetic alterations.
- Some hybrid oncocytic chromophobe tumors (HOCT) are associated with Birt-Hogg-Dubé syndrome (BHD) or renal oncocytosis, while others are sporadic. BHD can be confirmed by germline mutations in the FLCN tumor suppressor gene.
- Renal cell neoplasms with TFE3, TFEB and MITF rearrangements are now grouped together as MIT family translocation RCC.

**BRAIN TUMORS**

- Primary glioblastoma (GBM) may harbor 10q loss of heterozygosity (LOH) (70%), EGFR amplification (36%), CDKN2A deletion (31%) and PTEN mutations (25%), all of which denote aggressive disease.
- MGMT promoter hypermethylation (30%–40% of primary GBM) predicts improved response to alkylating agents and a lower risk of tumor recurrence.
- Associated with improved survival, IDH1 or IDH2 mutations are common in grades II and III astrocytoma, oligodendroglioma and secondary GBM, and uncommon in primary GBM. When present, they help to distinguish low-grade astrocytoma (~70%) from glioblastoma.
- Co-deletion of 1p / 19q (detectable by FISH / PCR / microarray) is characteristic of oligodendroglioma (Fig. 2), predicting response to conventional therapy. Equivocal FISH can be subsequently assessed by LOH analysis via PCR. ATRX loss of function mutations, which correlate well with negative IHC, favor diffuse astrocytoma over oligodendroglioma.
- Diffuse midline glioma is characterized by a K27M substitution in the histone H3 genes, H3F3A or HIST1H3B/HIST1H3C, resulting in hypomethylation and H3 protein inactivation. These tumors may also harbor mutations in TP53 (42%) or ACVR1 (24%) [4].
- BRAF fusion, most commonly with KIAA1549, is observed in 59%–90% of pilocytic astrocitomas.

**References**


**Meet the Authors**

Dr. Tsang joined the Pathology Outlines editorial board in 2019 and has been serving as Deputy Editor in Chief for Clinical Pathology for PathologyOutlines since 2020. She works as Chair of Pathology & Laboratory Medical Director at MedStar Washington Hospital Center in Washington, D.C. She has been practicing molecular pathology for more than a decade.

Dr. Chen is currently an Associate and Attending Pathologist at Geisinger Medical Center, where he participates in the clinical, academic and teaching services in surgical pathology and molecular diagnostics. He has been actively publishing and lecturing on topics related to molecular pathology of solid tumors.