Aneurysmal Bone Cyst
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Aneurysmal bone cyst (ABC) is a benign locally destructive bone neoplasm composed of multi-loculated blood-filled cystic spaces. The most common sites of involvement are the meta-diaphysis of the long bones and posterior elements of the vertebrae. Secondary, ABC-like changes can complicate a variety of other benign and malignant primary bone neoplasms, including giant cell tumor, fibrous dysplasia, and osteosarcoma. About two-thirds of primary ABCs have a rearrangement of the USP6 gene, which is not present in the ABC-like changes that occur secondary to other primary bone tumors (i.e., secondary ABC). Primary ABC of bone carries a variable but generally high rate of local recurrence. This paper provides an overview of the pathophysiology, clinical presentation, radiographic and pathologic findings, treatment, and prognosis of ABC.

Key Words: Bone cyst; Aneurysmal bone cyst; USP6 gene

Aneurysmal bone cyst (ABC) is a benign locally destructive bone neoplasm composed of multi-loculated blood-filled cystic spaces. It can present as a primary tumor, but ABC-like changes can also complicate other neoplastic diseases of bone.

ABC is a rare neoplasm with an annual prevalence of 0.32 per 100,000 young population [1], 0.14 per 100,000 general population [2], and comprising about 2.5% of all bone tumors [3]. It has an equal distribution among male and female patients [4,5], and most commonly is seen in skeletally immature patients especially in the first two decades of life [2].

Historically, it was thought that an ABC develops as the result of an underlying vascular event; increased venous blood flow; or a reaction to prior trauma [6]. However, in light of the recent molecular findings of the recurrent rearrangement involving the USP6 gene (chr.17p13.2 locus) [7-9], ABC is now considered a neoplasm rather than a reactive lesion.

Although ABC can affect any bone in the body, the craniofacial bones, vertebrae (particularly the posterior elements) and metaphysis of long tubular bones in the upper and lower extremities are more commonly involved [3]. Other less common sites of involvement include small tubular bones of hands and feet, tarsal bones, scapula, and pelvic bones.

Patients usually present with pain and swelling of variable duration at the site of involvement. Rarely, the initial presentation is pathologic fracture specifically in the major long tubular bones of the extremities [10]. In the vertebral lesions, symptoms of compression of the spinal cord or nerve roots may be the initial presentation [11,12].

The diagnosis of ABC requires the correlation of clinical, radiographic, and histologic findings and to distinguish the primary from a secondary form of the disease.

IMAGING

The radiographic features of ABC are quite distinct and aid in diagnosing the disease. Conventional radiographs show an eccentric radiolucent lesion with expansile remodeling of bone. A thin surrounding rim of the periostium and sub periosteal bone is usually present. The cyst wall trabeculae impart the multilocular appearance (Fig. 1). In the vertebral column, ABC most
commonly involves the posterior neural arch and can produce an eccentric “blowout” lesion [3]. In small tubular bones of hands and feet, the characteristic “finger-in-the-balloon” sign might be present.

Computed tomography shows a well-delineated lytic lesion, usually with a thin surrounding rim of reactive bone (Fig. 2A). Occasionally, fluid-fluid levels are visible. However, the best imaging modality to identify the fluid-fluid levels is magnetic resonance imaging. The cysts usually demonstrate variable signal intensity with a rim of low T1 and T2 signal. T1 post-contrast sequence may show some enhancement of septations (Fig. 2B–D) [13,14].

Isotope scan shows a peripheral uptake with central photopenia, which imparts a “donut sign” appearance. This appearance is not specific for ABC and can also be seen in other bone lesions with ABC-like changes such as chondroblastoma and giant cell tumor of bone.

**PATHOLOGY**

Grossly, an ABC appears as a well-demarcated spongy hemorrhagic lesion with variably-sized multiloculations. The cystic spaces are variable in size ranging from less than one millimeter to several centimeters [3]. It has irregular, sharply demarcated borders with a thin shell of reactive bone around it. A variable amount of solid component might be present, particularly in the...
digits (Fig. 3).

When encountered in an intraoperative consultation (frozen section), ABC is characterized by small fragments of cellular septa containing fibroblast-like stromal cells, osteoclast-like giant cells, and reactive woven bone that frequently displays osteoblastic rimming. While mitoses are typically easily identified, atypical mitoses and cytologic atypia are not present, and when these features are encountered on a frozen section they warrant a detailed discussion with the surgeon (Fig. 4).

ABC is almost always received as curetted material rather than an en-bloc resection. As a rule, the curetted material must be entirely submitted for histologic evaluation. Low-power microscopic examination shows a multiloculated cystic lesion with collapsed cyst walls within the background of blood and hemorrhage. The septa show mixed inflammatory cells, reactive fibroblasts, woven bone, and some vasoformative foci; however, the cyst walls lack a true lining. Characteristic calcified basophilic material, referred to as “blue reticulated chondroid-like material” might be present within the cyst walls [15]. The cysts are generally devoid of any lining but some flattened endothelial-like cells can be present. Osteoclast-type giant cells are found in clusters with increased numbers within the cyst wall. Mitoses can be easily identified, but atypical mitoses are not. Necrosis and cytologic atypia are not the features of ABC (Figs. 5, 6). There is no specific immunohistochemical stain for the diagnosis of ABC.

**MOLECULAR AND CYTOGENETICS**

Recurrent rearrangements of the short arm of chromosome 17 (p13.2) have been found in approximately 63% of cases of ABC [7]. This locus belongs to the ubiquitin-specific protease 6 (USP6), also known as Tre-2. Through promoter swapping, USP6 contributes its entire coding sequence as the 3' portion, and its transcription is substantially enhanced by replaceable 5' partner genes.

![Computed tomography (CT) and magnetic resonance imaging (MRI) of aneurysmal bone cyst. (A) CT scan shows a well-demarcated ovoid area of lucency within the medial aspect of the distal tibia. (B, C) MRI shows an expansile well-demarcated cystic lesion of the proximal tibia with fluid-fluid levels. (D) Sagittal view T2 MRI demonstrates multiple fluid-fluid levels of the cyst involving the calcaneus bone.](https://jpatholtm.org/https://doi.org/10.4132/jptm.2023.02.23)
As a result, the gene induces the production of matrix metalloproteinase activity via nuclear factor-kB. The most common fusion partner for the USP6 gene is CDH11 (about 30%); however, other genes such as TRAP150 (THRAP3), ZNF9 (CNBP), OMD, COL1A1, RUNX2, PAFAH1B1, CTNNB1, SEC31A, EIF1, FOSL2, STAT3, USP9X, ASAP1, FAT1, SARK1A, TNC, SPARC have been reported [17-19]. Rare cases of an unusually aggressive ABC with soft tissue extension and RUNX::USP6 fusion is also reported [20]. Rearrangement of the USP6 gene can be detected by fluorescence in-situ hybridization or fusion panel analysis such as targeted RNA sequencing.

Rearrangement of the USP6 gene has also been found in other soft tissue neoplasms; some with a limited capacity of bone formation, mimicking sarcomas; however, with limited growth potential and non-aggressive clinical course. These entities include nodular fasciitis, cellular fibroma of tendon sheath, myositis ossificans, and fibro-osseous pseudo tumor of digit. This common
finding suggests that these neoplasms might belong to a spectrum of disease processes that can best be referred to as USP6-associated neoplasms [16,21-24].

**DIFFERENTIAL DIAGNOSIS**

It is important to differentiate primary ABC from lesions with ABC-like changes. Such changes are more common in giant cell tumors of bone, fibrous dysplasia, chondroblastoma, osteoblastoma, and even osteosarcoma [4].
Radiographic findings often aid in narrowing down the differential diagnoses. As a general rule, any epiphyseal or diaphyseal-based lesion raises the possibility of ABC-like changes more than the primary ABC. For example, a diaphyseal lesion with multiple cysts and fluid-fluid levels indicates the possibility of telangiectatic osteosarcoma. The cystic epiphyseal-based lesions suggest the ABC-like changes in an underlying giant cell tumor of bone or chondroblastoma, depending on the patient’s skeletal maturity. In cases of giant cell tumor of bone with secondary ABC changes, immunohistochemical stain for H3F3A G34W (or other histone markers) can aid in diagnosis. The mononuclear cells of the giant cell tumor show nuclear immunoreactivity for the histone markers; however, the primary ABC does not demonstrate such a finding.

Distinction from telangiectatic osteosarcoma can be challenging due to some overlapping clinical and radiographic features. Unlike ABC, telangiectatic osteosarcoma shows highly atypical and anaplastic cells within the stroma, frequent atypical mitoses, and sometimes necrosis. Osteoid matrix production is minimal in these lesions. ABC shows frequent rearrangement of the USP6 gene; however, telangiectatic osteosarcoma does not show such findings. There is no specific immunohistochemical stain that aids in the differential diagnosis of these entities.

Central giant cell granuloma involves the gnathic bones and mimics “solid ABC.” It is usually solid with no or minimal cystic components. Cytologic atypia and necrosis are also not common. Unlike ABC, they lack rearrangement of the USP6 gene [25].

**TREATMENT AND PROGNOSIS**

Although the overall prognosis of ABC is good, the goal of any treatment modality is to slow down the disease progression, symptom relief, and prevention of pathologic fracture. En-bloc resection, although produces the least rate of disease recurrence [26,27], is not commonly performed due to an increased rate of functional impairment and morbidity [28]. Curretage with or without bone grafting is more commonly performed especially in anatomic locations amenable to surgical intervention.

Other therapeutic modalities including percutaneous doxycycline injection [29], arterial embolization, steroid or calcitonin injection, bisphosphonates, and RANKL inhibitors have been shown to be effective in the treatment of ABC in special clinical settings [30-35].

Local recurrence is seen in up to 1/3 of the cases, especially within the few months after the initial treatment, however, it is very rare after 2 years. Young age and open growth plates are also associated with an increased risk of local recurrence [36]. Rare cases of ABC with metastatic disease have also been reported [37].

**CONCLUSION**

Proper characterization of primary ABC of bone from ABC-like changes in other benign and malignant diseases requires the correlation of radiographic and pathologic findings. In challenging cases, molecular studies to identify the rearrangement of the USP6 gene will aid in diagnosis.

**Ethics Statement**

Not applicable.

**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.

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**Author Contributions**

Conceptualization: JDR, EN. Investigation: EN. Project administration: EN. Supervision: JDR, Visualization: EN, JDR. Writing—original draft: EN. Writing—review & editing: EN, JDR. Approval of final manuscript: EN, JDR.

**Conflicts of Interest**

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

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Significance of tumor-associated neutrophils, lymphocytes, and neutrophil-to-lymphocyte ratio in non-invasive and invasive bladder urothelial carcinoma

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Background: Tumor-infiltrating neutrophils and lymphocytes play essential roles in promoting or combating various neoplasms. This study aimed to investigate the association between tumor-infiltrating neutrophils and lymphocytes and the neutrophil-to-lymphocyte ratio in the progression of urothelial carcinoma. Methods: A total of 106 patients diagnosed with urothelial carcinoma were was. Pathological examination for tumor grade and stage and for tumor-infiltrating neutrophils, both CD4 and CD8\textsuperscript{+} T lymphocytes, as well as the neutrophil-to-lymphocyte ratio were evaluated. Results: The presence of neutrophils and the neutrophil-to-lymphocyte ratio correlated with high-grade urothelial neoplasms. In both low- and high-grade tumors, the lymphocytes increased during progression from a non-invasive neoplasm to an early-invasive neoplasm. CD8\textsuperscript{+} T lymphocytes increased in low-grade non–muscle-invasive tumors compared to non-invasive tumors. Additionally, there was a significant decrease in CD8\textsuperscript{+} T lymphocytes during progression to muscle-invasive tumors. Conclusions: Our results suggest that tumor-infiltrating neutrophils and CD8\textsuperscript{+} T lymphocytes have a significant effect on tumor grade and progression.

Key Words: Bladder urothelial carcinoma; Tumor-associated lymphocyte; Tumor-associated neutrophil; Neutrophil-lymphocyte ratio

Bladder cancer accounts for 3\% of global cancer diagnoses and is estimated to be the 10th most common cancer worldwide [1]. Urothelial carcinoma is the most common type of bladder cancer, constituting up to 95\% of bladder malignancies [2]. Most patients with urothelial carcinoma present with either non-invasive papillary neoplasms or superficially invasive tumors limited to the mucosa and submucosa. These neoplasms do not reach the muscle layer (i.e., they constitute non–muscle-invasive urothelial carcinoma [NMIUC; pTa or pT1]). For such neoplasms, transurethral resection of the bladder tumor (TURBT) is followed by adjuvant intravesical instillation therapy [3,4]. However, approximately 30\% of patients present with muscle invasion (i.e., muscle-invasive urothelial carcinoma [MIUC]; pT2) [5]. The five-year survival rate of NMIUC ranges from 50\% to 70\%, whereas that with MIUC, despite radical cystectomy and chemotherapy, is only 30\%–40\% [4,5]. Moreover, NMIUC progresses to MIUC in approximately 43\% of patients [6]. Thus, despite advancements in the management of bladder carcinomas, the outcomes have remained largely unchanged over several decades [7], highlighting the need to identify additional pathological parameters and biomarkers that could affect carcinogenesis and help improve management procedures [8].

The link between tumor microenvironment inflammation and tumor progression has been reported in several malignancies [9-11]. Neutrophils and lymphocytes are the main inflammatory cells observed in the tumor microenvironment [12]. Several studies
have highlighted the significance of elevated neutrophil-to-lymphocyte ratio (NLR) in patients with urothelial carcinoma and its association with a higher risk of recurrence and progression in NMIUC, as well as increased mortality and decreased overall survival in patients with MIUC [13,14]. Moreover, tumor-associated lymphocytes (TALs) exhibit diverse functions in various subsets. CD8 T lymphocytes are primarily responsible for attacking tumor cells [15,16]. Meanwhile, CD4 T lymphocytes are considered a double-edged immunologic sword: they can initiate and maintain CD8 lymphocyte anti-cancer immune responses [17] but can also convert anti-tumor activity to pro-tumor activity [18].

This study investigated the presence of tumor-associated neutrophils (TANs) and TALs, both CD4 and CD8, in both NMIUC and MIUC and their relation to well-known clinical and pathological prognostic parameters.

**MATERIALS AND METHODS**

**Patients and specimens**

This study included 106 patients pathologically diagnosed with urothelial bladder neoplasm by TURBT at Suez Canal University and Ismailia Oncology Hospitals from December 2021 to May 2022. Only cases with bladder urothelial neoplasms were selected. Clinical and pathological data of age, sex, tumor size (maximum diameter of the tumor) and number, pathological grade, and pTNM stage were recorded. The tumors were classified and graded according to the 2016 World Health Organization/International Society of Urological Pathology classification [19].

**Histopathological evaluation**

The samples were fixed with 10% formalin and embedded in paraffin. From each block, histological sections of 3-µm thickness were submitted, mounted to a glass slide, stained by hematoxylin and eosin, and reviewed to confirm the diagnosis of urothelial bladder neoplasm and to identify tumor grade, invasion depth, and presence of lympho-vascular invasion. Moreover, TANs and TALs were identified.

**Immunohistochemical staining**

Sections from the selected paraffin blocks were cut into 4-µm-thick sections for immunohistochemical (IHC) staining. Slides were prepared and incubated with primary anti-CD8 antibody (anti-CD8 alpha; ab4055, Abcam, Cambridge, UK) and anti-CD4 antibody (anti-CD4; ab133616, Abcam) to further subclassify the tumor-infiltrating lymphocytes (TILs). This was followed by incubation with the appropriate secondary antibody (anti-rabbit IgG; ab205718, Abcam). All slides were lightly counterstained with hematoxylin for 30 seconds prior to dehydration and mounting.

**Histopathological and IHC scoring**

TANs or TALs were defined as any neutrophils or lymphocytes that were in close proximity to the tumor base in non-invasive neoplasms or between tumor nests in invasive neoplasms. Four fields from the tumor were selected under low magnification (×100), and the neutrophils and lymphocytes were counted at high magnification (×400) using 2D image analysis software (Olympus CellSense, Tokyo, Japan) on an Olympus BX-46 microscope equipped with an Olympus SC30 digital camera. Care was taken not to count such inflammatory cells in areas of ulceration or erosion. Then, the average number was calculated and scored. TANs and TALs (either CD4 or CD8 T lymphocytes) were identified in the lamina propria just beneath the lower margin of the non-invasive urothelial neoplasm or infiltrated in the cancer nests or stroma.

**Statistical analysis and data interpretation**

Data were fed to a computer and analyzed using IBM SPSS Statistics for Windows ver. 27.0, released 2020 (IBM Corp., Armonk, NY). Qualitative data were described using number and percentage. The quantitative data were described using median (minimum and maximum) and interquartile range (IQR) for non-parametric data and mean and standard deviation for parametric data after determining normality using the Kolmogrov-Smirnov test. Significance of the obtained results was assigned at the (0.05) level.

**Data analysis**

Qualitative data were analyzed using chi-square test. If more than 25% of cells had a count less than 5 in the tables, the Monte Carlo test was used. Fisher exact test was employed when more than 25% of cells had a count less than five in 2 × 2 tables. The Mann-Whitney U-test was used to compare two independent groups.

**RESULTS**

The study included 106 patients with a median age of 58 years and an IQR of 49–66 years. There were 60 males (56.6%) and 46 females (43.4%) who were classified according to the degree of invasion into three groups (Table 1): group 1, non-invasive urothelial carcinoma (NIUC) (36 patients, 34%) (Fig. 1A);
Table 1. Numbers of tumor-associated neutrophils and lymphocytes and NLR in the studied groups classified according to tumor grade and state of tumor invasiveness

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>TAN (median)</th>
<th>TAL (median)</th>
<th>NLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading: Low</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-invasive neoplasms</td>
<td>12</td>
<td>5.5</td>
<td>13.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Non–muscle-invasive neoplasms</td>
<td>18</td>
<td>8.0</td>
<td>41.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Muscle-invasive neoplasms</td>
<td>12</td>
<td>4.0</td>
<td>35.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Grading: High</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-invasive neoplasms</td>
<td>24</td>
<td>11.5</td>
<td>12.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Non–muscle-invasive neoplasms</td>
<td>20</td>
<td>17.5</td>
<td>28.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Muscle-invasive neoplasms</td>
<td>20</td>
<td>30.0</td>
<td>25.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

NLR, neutrophil-to-lymphocyte ratio; TAN, tumor-associated neutrophils; TAL, tumor-associated lymphocytes.

Fig. 1. Tumor-associated neutrophils and CD8+ lymphocytes in non-invasive urothelial carcinoma. (A) Representative hematoxylin and eosin (H&E)-stained section of a low-grade papillary (arrows) non-invasive urothelial carcinoma. (B) Higher magnification of the base of the tumor reveals an abundance of lymphocytes (arrows) with few neutrophils (arrowheads). (C) Immunohistochemically, the lymphocytes express CD8 protein (arrows).

Fig. 2. Tumor-associated neutrophils and CD8+ lymphocytes in non–muscle-invasive urothelial carcinoma. (A) Representative hematoxylin and eosin–stained section of a lamina propria (non-muscle) invasive urothelial carcinoma. Tumor tissue grows as nests of malignant urothelial cells (arrows) infiltrating the lamina propria. (B) Higher magnification of the tumor stroma reveals an abundance of lymphocytes (arrows), with few neutrophils (arrowheads). (C) Immunohistochemically, many lymphocytes express CD8 protein (arrows).

group 2, non–muscle-invasive urothelial carcinoma (NMIUC) (38 patients, 35.8%) (Fig. 2A); group 3, MIUC (32 patients, 30.2%) (Fig. 3A).

The patients were further classified according to tumor grade: low-grade urothelial carcinoma (UC) (42 patients, 39.6%), high-grade UC (64 patients, 60.4%).

Significance of neutrophils

The presence of neutrophils correlated with high-grade urothelial neoplasms. Specifically, there was a significant increase in neutrophil number in high-grade UC cases compared to low-grade cases (p = .005) (Table 2).

We found a significant increase in the number of neutrophils in the MIUC (Fig. 3B) compared to the NIUC (Fig. 1B) in
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Fig. 3. Tumor-associated neutrophils and CD8+ lymphocytes in muscle-invasive urothelial carcinoma. (A) Representative hematoxylin and eosin–stained section of muscle-invasive urothelial carcinoma. Tumor tissue grows as nests of malignant urothelial cells (black arrows) infiltrating the muscle layer (red arrows). (B) Higher magnification of the tumor stroma reveals an abundance of neutrophils (black arrows) with few lymphocytes (arrowheads). Immunohistochemically (C), few lymphocytes express CD8 protein (arrowheads).

Table 2. Significance of tumor-associated neutrophils and lymphocytes, and NLR in the groups studied

<table>
<thead>
<tr>
<th>Group</th>
<th>TAN</th>
<th>TAL</th>
<th>NLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading: Low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-invasive neoplasms vs. non–muscle-invasive neoplasms</td>
<td>.851</td>
<td>.007</td>
<td>.152</td>
</tr>
<tr>
<td>Non–muscle-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.550</td>
<td>.315</td>
<td>&gt; .999</td>
</tr>
<tr>
<td>Non-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.460</td>
<td>.052</td>
<td>.128</td>
</tr>
<tr>
<td>Grading: High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-invasive neoplasms vs. non–muscle-invasive neoplasms</td>
<td>.570</td>
<td>.013</td>
<td>.232</td>
</tr>
<tr>
<td>Non–muscle-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.053</td>
<td>.913</td>
<td>.041</td>
</tr>
<tr>
<td>Non-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.012</td>
<td>.005</td>
<td>.792</td>
</tr>
</tbody>
</table>

Significant at p < .05.
NLR, neutrophil-to-lymphocyte ratio; TAN, tumor-associated neutrophils; TAL, tumor-associated lymphocytes.

high-grade neoplasms (p = .012). Similarly, there was a significant increase in neutrophils in the MIUC (Fig. 3B) compared to the NMIUC (Fig. 2B) in high-grade neoplasms (p = .053) (Table 2).

On the other hand, the number of neutrophils increased in the NMIUC (Fig. 2B) compared to the NIUC (Fig. 1B) in both low- and high-grade neoplasms; however, the increase was not statistically significant (p = .851 and p = .570, respectively) (Table 2).

Significance of lymphocytes

The presence of lymphocytes correlated more significantly with progressive invasion of urothelial neoplasms than with neoplasm grade. Nevertheless, there was a decrease in total lymphocyte count in high-grade neoplasms compared to low-grade neoplasms. However, the decrease was not statistically significant (p = .193).

Moreover, we identified a significant increase in lymphocytes during progression from NIUC (Fig. 1B) to NMIUC (Fig. 2B) in both low- and high-grade neoplasms (p = .007 and p = .013, respectively). Additionally, immunohistochemistry showed that the number of CD8+ lymphocytes in the low-grade neoplasms was significantly increased (p = .045) in the NMIUC group (Fig. 2C) compared to the NIUC group (Table 2, Fig. 1C). Furthermore, we found a significant increase in the lymphocyte count in the MIUC group (Fig. 3B) compared to the NIUC group (Fig. 1B) in both low- and high-grade neoplasms (p = .052 and p = .005, respectively) (Table 2).

On the other hand, when comparing MIUC (Fig. 3B) with NMIUC (Fig. 2B), there was a decrease in lymphocyte number, although it was not statistically significant in either low- or high-grade neoplasms (p = .315 and p = .913, respectively) (Table 2). However, there was a significant decrease in CD8+ lymphocytes in low-grade MIUC (Fig. 3C) compared to low-grade NMIUC (p = .052) (Table 3, Fig. 2C).

Significance of neutrophil-lymphocyte ratio

The NLR correlated with tumor grade; specifically, there was a significant increase in the ratio in high-grade urothelial neoplasms compared to low-grade neoplasms (p = .003) (Table 2). We found a significant increase in the NLR in the MIUC cases compared to the NMIUC cases in the high-grade neoplasms (p = .041), whereas we found no significant change in the ratio in the low-grade neoplasms (Table 2).
compared to low-grade cases (p = .005). There was also a significant increase in neutrophil count in high-grade UC cases correlated with high-grade urothelial neoplasms, as there was a significant increase in the number of neutrophils in MIUC cases compared to NIUC cases in high-grade neoplasms (p = .012). Similar-ly, there was a significant increase in neutrophil count in MIUC cases compared to NMIUC cases in high-grade neoplasms (p = .012). Similar-

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4+ T lymphocytes</th>
<th>CD8+ T lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading: Low</td>
<td>CD4+ T lymphocytes</td>
<td>CD8+ T lymphocytes</td>
</tr>
<tr>
<td>Non-invasive neoplasms vs. non–muscle-invasive neoplasms</td>
<td>.552</td>
<td>.045</td>
</tr>
<tr>
<td>Non–muscle-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.471</td>
<td>.062</td>
</tr>
<tr>
<td>Non-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.932</td>
<td>.812</td>
</tr>
<tr>
<td>Grading: High</td>
<td>CD4+ T lymphocytes</td>
<td>CD8+ T lymphocytes</td>
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<tr>
<td>Non-invasive neoplasms vs. non–muscle-invasive neoplasms</td>
<td>.864</td>
<td>.220</td>
</tr>
<tr>
<td>Non–muscle-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.425</td>
<td>.821</td>
</tr>
<tr>
<td>Non-invasive neoplasms vs. muscle-invasive neoplasms</td>
<td>.791</td>
<td>.335</td>
</tr>
</tbody>
</table>

Significant at p < .05.

Meanwhile, there was a non-significant decrease in the NLR in the NMIUC cases compared to the NIUC cases in both low- and high-grade neoplasms (p = .152 and p = .232, respectively) (Table 2). Similarly, there was a non-significant decrease in the NLR when comparing the NIUC group to the MIUC group in both low- and high-grade neoplasms (p = .128 and p = .792, respectively) (Table 2).

**DISCUSSION**

Inflammation within a tumor microenvironment plays an important role in tumor progression. Neutrophils are recognized for their antimicrobial activities and are found in many types of tumors. Early studies have suggested that TANs, which are short-lived, have no role in cancer progression. However, it has recently become evident that TANs with associated inflammation play a significant role in malignancy progression [20]. Neutrophils within tumor nests can induce anti-tumoral immune memory. Alternatively, they may have a pro-tumoral phenotype that promotes angiogenesis, invasion, metastasis, and immunosuppression [21]. This study revealed that the presence of neutrophils correlated with high-grade urothelial neoplasms, as there was a significant increase in neutrophil count in high-grade UC cases compared to low-grade cases (p = .005). There was also a significant increase in the number of neutrophils in MIUC cases compared to NIUC cases in high-grade neoplasms (p = .012). Similarly, there was a significant increase in neutrophil count in MIUC cases compared to NMIUC cases in high-grade neoplasms (p = .053).

The increased number of neutrophils in high-grade tumors and deeply invasive tumors may suggest that TANs are related to a poorer prognosis. Liu et al. [21] revealed similar results in their study on localized bladder cancer; i.e., an increased count of TANs was related to deeper tumor invasion and higher grade. This finding coincided with many studies on various tumor types in different organs, such as renal cell carcinoma, head and neck squamous cell carcinoma, and pancreatic adenocarcinoma [22-24]. Such pro-tumor activity of neutrophils could be attributed to released matrix of metalloproteinase 9, which frees vascular endothelial growth factor from the extracellular matrix to enhance angiogenesis, and to secreted arginase 1, which suppresses CD8 T lymphocytes. Moreover, TANs generate reactive oxygen species, which induce tumor progression [25,26]. Similarly, TALs have a dual regulatory role as they can induce an anti-tumor immune response by inhibiting tumor growth and tumor progression by creating a microenvironment that stimulates tumor outgrowth [27].

We found a significant increase in lymphocytes during progression from NIUC to NMIUC in both low- and high-grade neoplasms. This finding coincides with a previous study that reported an association between the adaptive immune response and tumor progression [28,29]. Regarding the lymphocyte population, we found that the number of CD8’ lymphocytes in low-grade neoplasms was significantly increased in the NMIUC group compared to the NIUC group. Similarly, Pichler et al. [30] and Mason-Lecomte et al. [31] reported an increased number of CD8’ lymphocytes in T1 bladder cancer compared to Ta bladder cancer. Moreover, we found a significant decrease in CD8’ lymphocytes in low-grade MIUC compared to low-grade NMIUC, indicating a possible role of CD8’ lymphocytes in hindering the progression to muscle invasion. This finding coincides with a recent review that evaluated the prognostic role of CD8’ TILs in cancer patients treated with immune checkpoint inhibitors. That review showed that CD8’ T cells at the invasive margin of tumors were negatively correlated with depth of invasion, and that high CD8’ TALs led to a 48% reduction in risk of disease progression compared with low CD8’ TILs [32].

On the other hand, Faraj et al. [33] found no significant relation between CD8-expressing T lymphocytes and any clinicopathological parameters; however, they did report a significant correlation with overall survival and disease-specific survival. Unlike our results, Hulsken et al. [34] found significantly higher
values for CD8+ T-cell infiltration in grade 3 tumors. They also found that increased CD8 expression was related to decreased survival and increased recurrence and was associated with poor prognosis. They only included specimens of patients diagnosed with T1 urothelial carcinoma, preventing comparison between tumor stages or relation of their findings to tumor progression [34]. Regarding CD4+ lymphocytes, we could not find a significant relation between their number and any of the groups studied. Similar results were obtained by previous studies, which could not show an association between CD4+ T cell density and any of the studied clinicopathological variables, including tumor stage and histological grade [35-37]. The relation between neutrophil counts in blood and lymphocytes has been correlated to clinicopathological parameters and has been suggested as a prognostic factor for urothelial carcinoma in many studies [38-40].

In the present study, we investigated the NLR at the tissue level. We found that the ratio correlated with tumor grade, as there was a significant increase in the ratio in high-grade urothelial neoplasms compared to low-grade neoplasms. We found a significant increase in this ratio in the MIUC cases compared to the NMIUC cases in high-grade neoplasms, confirming the tumor-promoting effect of neutrophils. It has been suggested that TANs are different from circulating neutrophils. Specifically, cytokines within the tumor microenvironment induce a population of neutrophils that have a pro-tumor phenotype that inhibits CD8+ T cells, and its tumor promotion increases with tumor progression [20]. However, Mandelli et al. [41] found no significant association between the tissue NLR and any of the studied clinicopathological variables, including tumor stage and grade. Such a discrepancy in the results highlights the complexity of the inflammatory response in urothelial carcinogenesis, and that other factors can potentiate or attenuate the role of inflammatory cells as anti- or pro-tumor cells. The importance of TALs is clearly demonstrated by the relation between elevated expression of programmed death-ligand 1 by tumor cells and higher TIL density, as well as by the association with higher histological grade and higher ptT category [42].

In summary, this study highlighted the significance of inflammatory cells within the tumor environment of bladder urothelial carcinoma. TANs correlated with tumor grade and stage, whereas TALs, especially CD8+ T cells, and the NLR were more likely to be associated with progression of tumor invasion rather than tumor grade. Further prospective multicenter studies with prolonged follow-up are recommended to confirm our results and to elucidate the prognostic role of inflammatory cells in the progression of urothelial carcinoma.

**Ethics Statement**

All procedures performed in the current study were approved by the Institutional Ethics Review Board of Suez Canal University (4687-5/12/2021) in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study. A copy of the written consent is available for review by the Editor-in-Chief of the journal.

**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.

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**Conflicts of Interest**

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

**Funding Statement**

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**References**


Cytopathology is important not only for disease diagnosis and healthcare but also for the Korean National Cancer Screening and Prevention Program [1,2]. The field of cytopathology has been a major component of pathology since its inception. The Korean Society for Cytopathology (KSC) was established nearly 40 years ago for quality control, development, and education in the field of cytopathology [3].

Although the KSC develops educational goals as a guideline for cytopathology education in Korea, there has been no systematic approach to understanding the current cytopathology training status for pathology residents. In addition, satisfaction with cytopathology education and with the outcomes of the current training/educational program has not been investigated in Korea. There is also a concern that the current training program for pathology residents does not focus sufficiently on cytopathology, especially because the field is so clinically diverse and continues to play an important role in patient management and cancer screening.

As an initial step to ensure higher standards of cytopathology training, we conducted a survey among pathology residents and pathologists to obtain their views on the current status of cytopathology education and training. With the support of the KSC, this research aimed to obtain comprehensive data on the current state of cytopathology education for residents and to evaluate
educational outcomes as a baseline evaluation for further improvement of cytopathology training in Korea.

**MATERIAL AND METHODS**

**Survey participants and implementation**

The survey was conducted among 988 board-certified pathologists and 87 training residents registered as members of the KSC. A series of questions addressing cytopathology residency training were developed, edited, and revised according to the KSC mission on clarity, quality, appropriateness, and importance. In December 2020, we conducted an online, confidential survey of KSC members. The questionnaire was uploaded onto an online survey site. KSC members were invited by email to participate, and an additional invitation was posted on social media for non-responsive residents.

**Survey instrument and statistical analysis**

The survey questionnaire included both closed- and open-ended questions. The questionnaire was divided into the following five sections: (1) general information, (2) current resident cytopathology training curriculum, (3) degree of resident satisfaction with the cytopathology training curriculum, (4) performance prediction and directions for improvement, and (5) training transfer. A total of 62 questions were included in the survey. Section 3 was filled out only by the pathology residents. These questions were rated on a 5-point Likert scale (5, strongly agree; 4, agree; 3, neutral; 2, disagree; and 1, strongly disagree). Residents who responded with 3, 4, or 5 were considered “satisfied”, and those who responded with 1 or 2 were considered “unsatisfied”. There were also open-ended questions about why responders were unsatisfied with the training curriculum and suggestions for improvements were requested (see Supplementary Data S1 for full survey and response statistics). Descriptive statistics were used to summarize demographics and outcome measures.

**RESULTS**

**General information**

The response rate for the survey was 12.3% (n=132/1,075). The respondents were 32 training residents (24.2%) and 100 board-certified pathologists (75.8%). The response rate among the residents was 36.8% (n=32/87). However, one of the resident responders did not participate in section 3; therefore, the total number of responses to this section was 31. Twenty-five of the board-certified pathologists were currently directors of the departments where they worked. By practice experience, 57% of the pathologists had more than 16 years, 9% had 11 to 15 years, 21% had 6 to 10 years, and 13% had less than 5 years. Furthermore, 29% of their institutions had residents in training at the time of the survey.

**Current resident cytopathology training curriculum**

This section was answered by residents and pathologists who had pathology residents in their institution. The proportion of respondents who answered that there was no training period exclusively dedicated to cytopathology was 52.5%. The proportion of those who had more than 3 months of cytopathology training was 36.1%. Regarding the percentage of training time allocated to cytopathology training or work time, 49.2% answered that cytopathology training comprised 10%–25% of the training period. The beginning of cytopathology education was most often in the second year of residency (59.0%), followed by the first (31.1%) and third years (9.8%). Most hospitals (63.9%) did not further divide the training program according to specific cytopathology fields (e.g., gynecologic vs. non-gynecologic). Of the respondents, 77.1% answered that the frequency of diagnosing cytopathology slides (i.e., signing-out) with the residents was more than 4 times per month. The following knowledge and skills were studied in Korean pathology departments: (1) screening of marked cytopathology slides, 85.2%; (2) preparing draft reports, 80.3%; (3) attending onsite rapid assessment of fine needle aspiration (FNA) or endobronchial ultrasound FNA, 44.3%; (4) screening of unmarked cytopathology slides, 41.0%; and (5) performing FNA, 1.6%. The majority of the institutions did not have designated conference time for cytopathology education (78.7%). In terms of the frequency of cytopathology education sessions, in 47.5% they occurred less than 3 times a year, and 31.1% reported none each year. Furthermore, regarding the frequency with which residents attended cytopathology conferences, seminars, or workshops held by the cytopathology society or other external organizations, 67.2% attended 2–6 times a year.

In the open-ended question, respondents were asked to express their opinion on the current cytopathology training program in Korea. Overall, 12% (16/132) of the respondents left comments, and the most common opinion was that the cytopathology society needed to organize more educational programs, especially further implementation of online education (from 10 respondents) (see Supplementary Data S1 for the other responses).
Degree of resident satisfaction with the cytopathology training curriculum

This section was answered only by residents who were in training at the time of the survey. This section asked questions that concerned three areas: (1) overall satisfaction with the cytopathology training program, (2) satisfaction with the training practices and specific training fields, and (3) satisfaction with the training environment. The mean overall satisfaction with the cytopathology training program was 3.1 (Fig. 1). Satisfaction with training practice and specific training fields ranged from 2.7 to 3.5 (Fig. 2). Sign-out with a senior pathologist was rated as the most satisfactory field of the training (average rating, 3.5). Training and on-site assessment of FNA specimens was rated as the most unsatisfactory field of the training (average rating, 2.7). Pathology residents rated satisfaction with the cytopathology training environment (e.g., number of teaching pathologists, reference bibliographies, and teaching or reference slides) as 3.2–3.3 (Fig. 3).

Performance prediction and directions for the improvement

All the respondents answered questions in this section. We first asked whether practicing pathologists and residents were familiar with the current cytopathology training guidelines that were established by the KSC [4]. According to the survey results, 41.7% of the participants acknowledged being aware of the guidelines, albeit not entirely familiar, while 46.2% reported not knowing the guideline. When the residents were asked whether the current education and training system could help them accomplish the training goals suggested by the KSC guidelines, 44.7% were neutral and 32.5% answered that they were likely to accomplish the training goal. Among the 42 responders who did not feel they would gain the ability to competently make a cytopathology diagnosis upon completion of training, lack of specific curriculum and training time (57.1%) and lack of diverse cytopathology cases during training (23.8%) were selected as the main reasons.

<table>
<thead>
<tr>
<th>Questionnaire items</th>
<th>Frequencies of satisfaction scores</th>
<th>Average of satisfaction scores</th>
<th>Percentage of satisfaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1. I am satisfied with the current overall cytopathology training</td>
<td>1, strongly disagree 2, disagree 3, neutral 4, agree 5, strongly agree</td>
<td>3.1</td>
<td>71.0%</td>
</tr>
<tr>
<td>Q2. I am satisfied with the current overall gynecologic cytopathology training</td>
<td>3.9</td>
<td>87.1%</td>
<td></td>
</tr>
<tr>
<td>Q3. I am satisfied with the current overall non-gynecologic cytopathology training</td>
<td>3.1</td>
<td>64.5%</td>
<td></td>
</tr>
<tr>
<td>Q4. I am satisfied with the training hours occupied by the cytopathology during pathology resident training period</td>
<td>3.0</td>
<td>71.0%</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Overall satisfaction ratings provided by residents. *31 Responders answered.

<table>
<thead>
<tr>
<th>Questionnaire items</th>
<th>Frequencies of satisfaction scores</th>
<th>Average of satisfaction scores</th>
<th>Percentage of satisfaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q5. I am satisfied with the training practice - slide screening and preparing draft reports</td>
<td>3.3</td>
<td>77.0%</td>
<td></td>
</tr>
<tr>
<td>Q6. I am satisfied with the training practice - sign-out and reports to diagnose with pathologist</td>
<td>3.5</td>
<td>61.3%</td>
<td></td>
</tr>
<tr>
<td>Q7. I am satisfied with the training practice - institutional education sessions (e.g., lectures)</td>
<td>2.8</td>
<td>71.0%</td>
<td></td>
</tr>
<tr>
<td>Q8. I am satisfied with the education on criteria and guidelines</td>
<td>3.0</td>
<td>67.7%</td>
<td></td>
</tr>
<tr>
<td>Q9. I am satisfied with the case conferences</td>
<td>2.9</td>
<td>77.4%</td>
<td></td>
</tr>
<tr>
<td>Q10. I am satisfied with the education and conferences provided by society or other institutions</td>
<td>3.2</td>
<td>64.5%</td>
<td></td>
</tr>
<tr>
<td>Q11. I am satisfied with the training on FNA and on-site rapid assessment</td>
<td>2.7</td>
<td>61.5%</td>
<td></td>
</tr>
<tr>
<td>Q12. I am satisfied with the training on quality control and laboratory management</td>
<td>3.0</td>
<td>77.4%</td>
<td></td>
</tr>
<tr>
<td>Q13. I am satisfied with the training on digital pathology and automated screening system</td>
<td>2.8</td>
<td>64.5%</td>
<td></td>
</tr>
<tr>
<td>Q14. I am satisfied with cytopathology research participation</td>
<td>2.8</td>
<td>64.5%</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Satisfaction with cytopathology training practices and specific training fields. FNA, fine needle aspiration. *31 Responders answered.
The second group of questions was about fields of training that needed strengthening. Among gynecologic and several non-gynecologic fields, gynecologic cytopathology and thyroid FNA were chosen as the training sectors that needed to be strengthened (average rating, 3.8) (Fig. 4).

The third group of questions was about the predicted performance of the cytopathology training. The respondents expected that the current cytopathology training system would most likely enhance cytopathology diagnostic skills (average rating, 3.4) but was unlikely to enhance understanding and skills regarding digital pathology and automated screening systems (average rating, 2.8) (Fig. 5).

In the open-ended question, respondents expressed the need for more exposure to diverse specimens, the need for an improved cytopathology training program, and continuing education for the pathologists who train residents. More opinions are provided in Supplementary Data S1.

**Training transfer**

Respondents mostly stated that they applied what they learned during cytopathology training in real-world practice (average rating, 3.7), that their cytopathology training enhanced their performance at work (average rating, 3.7), and that they have tried to apply what they learned in the training process to their actual work tasks (average rating, 3.8). Also, most residents stated that the cytopathology training program has enhanced their job expertise (average rating, 3.7) (Fig. 6).

**DISCUSSION**

This study is the first survey addressing the current status and perspectives on cytopathology training in Korea. Globally, reports or surveys on the status of cytopathology training are scarce [5-8]. Based on the survey results, most institutions did not have an exclusive cytopathology training period for the residents (52.5% of respondents), and the training period was approximately 10%–20% of the workload. Mostly, the training comprised drafting a diagnosis in cytopathology cases (80.3%) that was prescreened and marked by cytotechnicians (85.2%). The residents had sign-out sessions with board-certified pathologists once (27.9%) or more than twice (49.2%) per week. There were very few intra-institutional cytopathology conferences or educa-

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**Fig. 3.** Satisfaction with the training environment. *31 Responders answered.

**Fig. 4.** Areas of cytopathology training that should be strengthened. *132 Responders answered.
tion sessions, less than three sessions per year.

The overall satisfaction rating on cytopathology education was 3.1 among the pathology residents. In specific fields, sign-out sessions with board-certified pathologists were rated as the most satisfactory of the training program (average rating, 3.5), while training on the FNA technique was rated as the least satisfactory (average rating, 2.7). We found that the traditional training approach, which requires enhancing the experience through co-sign-out of the diagnosis and is often called apprenticeship education, was satisfactory and necessary. However, there seems to be little training time or education resources allocated to cytopathology training. The reality is that many board-certified

<table>
<thead>
<tr>
<th>Questionnaire items</th>
<th>Frequencies of satisfaction scores *</th>
<th>Average of agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1. Current cytopathology training will improve cytopathology diagnostic skills</td>
<td>4.5%</td>
<td>7.6%</td>
</tr>
<tr>
<td>Q2. Current cytopathology training will improve the ability to analyze differences between histologic and cytopathologic findings</td>
<td>5.3%</td>
<td>7.6%</td>
</tr>
<tr>
<td>Q3. Current cytopathology training will improve communication skills with colleagues, including clinicians</td>
<td>6.1%</td>
<td>9.1%</td>
</tr>
<tr>
<td>Q4. Current cytopathology training will improve the ability to apply ancillary staining methods in cytopathology</td>
<td>4.5%</td>
<td>12.1%</td>
</tr>
<tr>
<td>Q5. Current cytopathology training will improve the understanding of pre-analytical conditions</td>
<td>5.3%</td>
<td>15.9%</td>
</tr>
<tr>
<td>Q6. Current cytopathology training will improve basic knowledge on cytopathology</td>
<td>4.5%</td>
<td>12.9%</td>
</tr>
<tr>
<td>Q7. Current cytopathology training will improve research ability in cytopathology</td>
<td>9.1%</td>
<td>20.5%</td>
</tr>
<tr>
<td>Q8. Current cytopathology training will improve quality control and laboratory management abilities</td>
<td>5.3%</td>
<td>15.9%</td>
</tr>
<tr>
<td>Q9. Current cytopathology training will improve understanding and use of diagnostic systems, such as, the cervical pap smear Bethesda system</td>
<td>5.3%</td>
<td>7.6%</td>
</tr>
<tr>
<td>Q10. Current cytopathology training will improve understanding and use of diagnostic systems, such as, the thyroid Bethesda, salivary Milan, and urinal Paris systems</td>
<td>3.8%</td>
<td>6.8%</td>
</tr>
<tr>
<td>Q11. Current cytopathology training will improve understanding and use of digital pathology and automated screening system</td>
<td>15.1%</td>
<td>20.5%</td>
</tr>
<tr>
<td>Q12. Current cytopathology training will improve the use of molecular cytopathology</td>
<td>12.1%</td>
<td>21.2%</td>
</tr>
<tr>
<td>Q13. Upon completion of a four-year training, the residents will have the ability to independently and competently make a cytopathology diagnosis.</td>
<td>10.6%</td>
<td>21.2%</td>
</tr>
</tbody>
</table>

Fig. 5. Performance prediction. *132 Responders answered.

<table>
<thead>
<tr>
<th>Questionnaire items</th>
<th>Frequencies of satisfaction scores *</th>
<th>Average of agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1. I am using what I learned in cytopathology training in real-time work</td>
<td>2.3%</td>
<td>5.3%</td>
</tr>
<tr>
<td>Q2. What I learned during cytopathology training has improved my performance</td>
<td>3.0%</td>
<td>3.8%</td>
</tr>
<tr>
<td>Q3. I am applying what I have learned in the cytopathology training to real work</td>
<td>3.8%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Q4. After the cytopathology training, I receive positive evaluation from the seniors or colleagues about my improved work performance</td>
<td>3.8%</td>
<td>7.6%</td>
</tr>
<tr>
<td>Q5. What I learned during the cytopathology training helped me solve problems in real-time work that deals with patients</td>
<td>3.0%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Q6. Cytopathology training has improved my job expertise</td>
<td>3.0%</td>
<td>2.3%</td>
</tr>
<tr>
<td>Q7. Cytopathology training has improved my communication skills with the patients and clinicians</td>
<td>5.3%</td>
<td>6.1%</td>
</tr>
</tbody>
</table>

Fig. 6. Training transfer. *132 Responders answered.
fied pathologists have an excessive workload; thus, it may be difficult for them to dedicate additional time for residency training and co-sign-out. Our study underlines the importance of education through personal teaching and a co-sign out approach; therefore, a systematic effort to provide sufficient educational opportunities is needed.

The field of education that needs to be strengthened the most is gynecologic cytopathology and thyroid FNA cytopathology (average rating, 3.8). According to nationwide survey data, gynecology and thyroid FNA are the most frequently performed cytopathology examinations [3]. The reason they were selected as an area that needed to be strengthened may be because of the high demand for those areas in real-time diagnostic work. Generally, most residents expected that they would be able to work independently and proficiently diagnose cases related to cytopathology after completing residency training (average rating, 3.0). However, many opinions have suggested that the lack of specific cytopathology training curricula and training time may affect residents' competence in cytopathology. A lack of training time was noted in a European cytopathology training survey [6]. The KSC developed training performance and goals for pathology residents, but few pathologists and residents were familiar with these guidelines (average rating, 2.5). It is necessary to reflect on the extent to which these guidelines have been used in individual institutions.

Even though there were many opinions that highlighted the lack of education and training time for cytopathology, the respondents rated training transfer questions as 3.0 or more. This suggests that cytopathology training has been useful and effective in the daily work of the respondents. Cytopathology training will progress if the results from this survey are reflected in future training development.

The low response rate is the major limitation of this survey. The response rate among residents (36.8%) was higher than that of board-certified pathologists (10.1%), but the responses may not fully reflect the opinions of all residents.

In this survey, there were several suggestions for improving cytopathology education in the future that seemed necessary. First, the respondents suggested that a systematic residency training program should be implemented at each institution. It is necessary to consider the strengths and weaknesses of the current training system and to reinforce areas with low satisfaction. Second, they suggested that guidelines for a systematic residency training program should be provided by the KSC. The goals and guidelines of current residency training state the depth of experience in diagnosis and fields that the residents should be trained in. However, there is no specific action plan, such as how the residents should be taught or the minimum training period required. This guide does not need to be regulatory or mandatory; however, it will still be helpful to each institution that attempts to improve their cytopathology training program [7,9]. Currently, the qualification requirement for pathology board certification does not include minimum educational participation hours in the field of cytopathology. Creating such a requirement could be one approach to increasing educational opportunities, such as intradepartmental cytopathology conferences or society education, and to encourage participation of residents in these educational opportunities. Lastly, the respondents suggested that more diverse educational programs should be provided by the academic society. Online education, case galleries, and clinical case-oriented education sessions were suggested as solutions. This survey was conducted at a time when most academic events were suspended due to the coronavirus disease 2019 (COVID-19) pandemic. Therefore, the lack of educational opportunities reported may have been due to the COVID-19 situation. With these results, we hope that institutions and the KSC can develop more diverse educational programs, such as an on-demand online educational program. The KSC is currently developing an online educational site that reflects the study suggestions, and the site will be launched soon.

This study is expected to improve the quality of cytopathology education, provide opportunities to standardize cytopathology education for residents, and to serve as a basis for improvement in cytopathology education for both residents and practicing pathologists. We expect that this study will inform systematic training for competent medical personnel armed with broad knowledge of cytopathology and strong problem-solving abilities.

**Supplementary Information**

The Data Supplement is available with this article at https://doi.org/10.4132/jptm.2023.01.06.

**Ethics Statement**

All procedures performed in the current study were approved by the Institutional Review Board of St. Vincent's Hospital (VC20QCDI0083) in accordance with the 1964 Helsinki Declaration and its later amendments. The need for formal written informed consent was waived by the institutional review board.

**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.
Author Contributions
Conceptualization: HJC, TJK. Data curation: UC. Formal analysis: UC. Funding acquisition: HJC. Investigation: HJC, UC. Methodology: HJC, UC, TJK. Project administration: HJC. Resources: UC. Software: UC. Supervision: HJC. Validation: HJC, UC. Visualization: HJC, UC. Writing—original draft: UC. Writing—review & editing: HJC, TJK, WSK, KYL, HKY. Approval of final manuscript: all authors.

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

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References
Since the declaration of coronavirus disease 2019 (COVID-19) as a pandemic, several parameters have been used to measure its public health impact [1]. These statistical measures include the rate of detected cases, rate of critical cases, and importantly the rate of death as a result of the disease [2]. The case fatality ratio (CFR) can be used to evaluate the severity among the detected disease such as COVID-19 [3-5] which can lead to an under- or overestimation of the CFR [3-5].

Autopsy examination is the definitive method to establish the cause of death, particularly in the context of a novel infectious disease such as COVID-19 [6,7]. It can be chiefly beneficial to identify the causal relationship between death and infectious diseases in pandemic setting [8]. In addition, autopsy examination can provide important clues to understand the mechanisms of SARS-CoV-2–induced organ damage and its underlying mechanisms [9]. Forensic examination is a central step in a multidisciplinary approach to determine the cause of death in medicolegal
cases positive for COVID-19 [10,11]. Thus, autopsy examination is invaluable in resolving disputes in medicolegal cases with legal liability of health professionals in the context of COVID-19–related deaths, especially in the hospitals and nursing homes [12,13]. The utility of autopsy examination has also been emphasized in cases of breakthrough infection post–COVID-19 vaccination [10]. In the absence of suitable precautionary measures, autopsy examination can be done using the minimally invasive approach, which can provide important clues to data on deaths attributed to COVID-19 [14].

More than 2 years have passed amid COVID-19 pandemic, and much has been known regarding its epidemiology, pathophysiology, and clinical manifestations [15]. Specifically, it is well-known now that there is a wide variability in clinical presentation of the disease with a majority of cases being asymptomatic or mild-to-moderate in clinically apparent infections [16]. Nevertheless, a substantial number of COVID-19 cases evolve into severe or critical disease with respiratory failure, septic shock, and/or multiple-organ failure and death from the disease [17,18].

Despite the rapid availability of extensive and comprehensive research on COVID-19, several aspects of the disease have not been fully elucidated yet, one of which is labeling the deaths with positive SARS-CoV-2 testing results as COVID-19–related mortalities despite the absence of typical findings of the disease [11,19,20]. Thus, autopsy examination can be the sole method to reach a definitive conclusion about the genuine cause of death in such a scenario [21].

In COVID-19, the causative agent is SARS-CoV-2, which utilizes angiotensin-converting enzyme 2 receptors to enter the target cells [22]. Although COVID-19 is primarily a respiratory disease, extrapolummary manifestations including cardiovascular effects are commonplace especially in severe and critical cases [23]. Hypercoagulability state in association with COVID-19 has been observed frequently and could be linked to dual effect of direct virus damage and activation of various host inflammatory mediators [24]. Subsequently, the hypercoagulability can give rise to acute coronary syndrome (ACS) as a cardiac manifestation of the disease [25].

For the diagnosis of COVID-19, molecular detection by nucleic acid amplification testing including real-time polymerase chain reaction (qPCR) can be considered the gold standard laboratory diagnostic method [26]. Another diagnostic approach, which can be helpful in COVID-19 cases with late presentation include serologic testing for IgM antibodies [27]. The use of electron microscopy (EM) is considered a reliable tool to delineate the ultra-structural details of viruses, including SARS-CoV-2; however, its use in clinical practice has been limited by cost issues besides the need for technical expertise and tedious procedure compared to molecular detection techniques [28].

Histopathologic findings in autopsies from COVID-19–positive cases include the detection of diffuse alveolar damage (DAD) in a vast majority of cases [28,29], with frequent detection of microthrombi. The spectrum of findings ranges from alveolar hyaline membranes formation, interstitial edema, necrosis of type 1 pneumocytes and endothelial cells in the exudative phase to hyperplasia of type II pneumocytes, and interstitial myofibroblasts with lymphocytic infiltration in the organizing phase and eventually collagenous fibrosis and end-stage lung changes in the fibrotic phase [29]. Despite the view that COVID-19 pneumonia is a heterogeneous disease, from a histopathologic point of view, there is previous evidence that acute and organizing DAD can be considered the primary cause of mortality due to SARS-CoV-2 infection [29,30].

In spite of the growing number of case series describing the autopsy findings in COVID-19–positive deaths, there is a general lack of reports describing the pathophysiology and histopathologic examination of autopsies in the Middle East region to the best of our knowledge [6,7,31]. Thus, such an investigation can be considered necessary to supplement the previous literature with more insights that can be helpful to better define the histopathologic changes that occur in the context of COVID-19 and to establish the cause of death in cases with atypical presentation. Therefore, the current study aimed to evaluate the histopathologic findings in autopsies in a case series from individuals who were positive for COVID-19 at time of death in Jordan.

MATERIALS AND METHODS

Study design

The current study was based on conducting a serial postmortem examination in COVID-19–positive patients at time of death among medicolegal cases in which autopsies are mandatory by Jordanian law to determine the cause of death. Testing for SARS-CoV-2 for all cases of death was mandatory by law before burial in Jordan. Autopsies were conducted at Jordan University Hospital (JUH) and Zarqa Hospital. Forensic gross assessment was done for specimens from the lungs, heart, among other organs. Postmortem specimens were tested for SARS-CoV-2 by qPCR, serologic testing, or both. The final confirmation of SARS-CoV-2 detection in autopsies was done through examining lung specimens under EM, which was conducted at the Department of Pathology, Microbiology and Forensic Medicine at the School of Medicine at the University of Jordan.
of Medicine, University of Jordan.

Testing for COVID-19

The postmortem detection of SARS-CoV-2 was based on three approaches as follows: (1) qPCR of nasopharyngeal swabs, with RNA purification using the automated Zybio Nucleic Acid Isolation System EXM3000 (Zybio, Chongqing, China), with reverse-transcriptase qPCR being done using SARS-CoV-2 Nucleic Acid Detection Kit (Zybio) targeting three genomic regions (Envelope, ORF1ab, and Nucleocapsid) with interpretation according to manufacturer's instructions; (2) serologic testing to detect IgM/IgG; (3) EM examination of autopsies with lung tissue.

Initially, all cases were identified through either a positive qPCR testing result or serologic testing, while EM examination was conducted among all cases.

Autopsy examination

Autopsies carried out on COVID-19–positive dead bodies were done by authority of the District Attorney since all cases were labeled as medicolegal cases. Such cases are considered medicolegal due to the sudden and unexpected death with no clear causes, with consent to conduct autopsy examination being waived in such cases. Most included autopsies were limited to the chest and abdomen, but some cases were examined fully according to the circumstances of their death. Personal protective equipment was used among all staff members. The procedures involved the minimum needed number of staff. Full external inspection of the body as it was received was conducted initially, followed by midline chest and abdomen incision with extraction of the lungs and heart with full inspection and documentation by “inspection report and photographing of all positive findings.” Tissue sampling was limited to the lungs and heart.

Histopathologic examination

Tissue specimens were fixed in 10% formalin for 72 hours, dehydrated by ethanol, and cleared twice by xylene, then embedded in paraffin, and sectioned as 5 μm sections. The prepared sections were dried and stained by hematoxylin and eosin full final histopathologic examination, which was conducted by the first authors and the senior author (M.A. and M.A.A.-A.), who are consultant histopathologists at JUH.

The differentiation between “death from COVID-19” vs. “death with SARS-CoV-2 infection” was based on the presence of DAD in the former group in contrast to its total absence in the later group.

EM examination

The procedure of EM was adopted from a previous publication by Shatarat et al. [32]. Briefly, the extracted tissues from autopsies were fixed for one hour in buffered 1%–2% osmium tetroxide followed by gradual dehydration in ethanol/propylene oxide and embedding in epoxy resin media mixture. The U7 ultra-cut was used to obtain sections with a range of 70–90 nm, followed by mounting on 200 mesh copper grids, and contrasted with uranyl acetate and lead citrate [32]. The FEI Morgagni Transmission Electron Microscope was used for image acquisition and analysis.

Criteria for identification of “death from COVID-19” vs. “death with SARS-CoV-2 infection”

As previously stated, we used histopathologic findings as the sole criterion to define death from COVID-19 if DAD was found while its absence, was used to denote death with SARS-CoV-2 infection.

Statistical analysis

Based on the small sample size, two-sided Fisher exact test was used to investigate categorical variables, while assessment of the association between age as a continuous variable with dichotomous categorical variables was done using the Mann-Whitney U test. Statistical analysis was done using IBM SPSS Statistics for Windows ver. 22.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Characteristics of the included cases

The study cohort comprised 17 individuals who were positive for COVID-19 at postmortem SARS-CoV-2 assessment by qPCR testing of nasopharyngeal swabs (n = 4), serologic testing for SARS-CoV-2 IgM (n = 3) or confirmed by both methods (n = 10). Two individuals were diagnosed with COVID-19 by qPCR several days prior to death (5 days and 12 days antemortem), whereas the remaining cases had positive test results within 24 hours from being declared dead. The majority of cases were males (76.5%), Jordanians (70.6%), and the average age at time of death was 50 years (Table 1).

The majority of cases were declared dead at home (n = 11, 64.7%). Eight cases were diagnosed during the first COVID-19 wave in Jordan, while the remaining nine cases were diagnosed during the second wave of COVID-19 in the country.

The most frequent co-morbidities in the study cohort were hypertension and obesity (n = 5 for both). Previous emergency
Department visits were recorded for eight cases (four of which were for respiratory symptoms including shortness of breath and cough).

Four cases died with SARS-CoV-2 infection compared to 13 dying from COVID-19

Evidence of the presence of SARS-CoV-2 with the absence of DAD was found in four cases (23.5%). Based on that, the case series was divided into two groups “death with SARS-CoV-2 infection” vs. “death from COVID-19.” In the former group, two cases were presumptively diagnosed with pneumonia at death, one with ACS and the last case was diagnosed with subdural and subarachnoid hemorrhage. For the “death from COVID-19” group, six were diagnosed with pneumonia, four were diagnosed with pulmonary embolism, and three were diagnosed with acute coronary syndrome. The mean age of individuals within the “death with SARS-CoV-2 infection” was younger than the mean age of those within the “death from COVID-19” group (39 years vs. 54 years, p = .078, Mann-Whitney U test).

Absence of co-morbidities was recorded among all cases in the “death with SARS-CoV-2 infection” (0/4) in contrast to (9/11, 81.8%) having at least one co-morbid condition in the “death from COVID-19” group with missing data in two cases (p = .011, Fisher exact test).

Autopsy findings from lung tissues

Gross examination of the lungs in the study cohort revealed the common occurrence of congestion and edema followed by hepatization of lungs (Table 2).

On histopathologic examination, DAD was detected in 13/17 cases (76.5%), which was sub-classified into exudative DAD (n = 5), proliferative DAD (n = 7), and a single case of evolving fibrosing DAD. All cases with exudative DAD showed hyaline membrane formation, denudation and/or necrosis of type I pneumocytes (Fig. 1).

Variable degrees of hyaline membrane organization and type II pneumocytes hyperplasia were noted in the seven proliferative DAD cases (Fig. 2).

A majority of cases displayed evidence of interstitial and intra-alveolar edema (64.7%), lymphocytic infiltration of the alveoli (64.7%), and pulmonary capillaries congestion (100%). Microthrombi were found in eight cases (47%), of which two cases showed no DAD, two cases displayed exudative DAD, and four cases showed proliferative DAD. Empysematous changes were noted in eight cases (Table 2, Fig. 3).

In the absence of co-morbidities, no specific histologic changes were noticed in alveoli (n = 4), compared to the presence of lymphocytic infiltration in 8/9 of the cases with co-morbidities (p = .011, Fisher exact test). All cases in the “died with COVID-19” group lacked alveolar changes by definition (n = 4), compared to 11/13 with lymphocytic infiltration in the “died from COVID-19” group (p = .006, Fisher exact test).

Autopsy findings from heart tissues

Gross examination of the heart in this case series revealed the relatively high prevalence of atherosclerotic changes in coronary arteries (n = 8, 47.1%). Histopathologic changes were scarcely detected. Fibrosis in intima and thickening of media were detected in a single case, while myocardial cell necrosis was found in another single case (Table 2).

EM findings

All cases were examined using EM, and spherical structures with surface spikes were found in all cases (n = 17, 100%) (Fig. 4).

Table 1. Summary of the clinical features of the study cohort (n = 17)

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range, yr)</td>
<td>50 (27–77)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13 (76.5)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>Nationality</td>
<td></td>
</tr>
<tr>
<td>Jordanian</td>
<td>12 (70.6)</td>
</tr>
<tr>
<td>Non-Jordanian</td>
<td>5 (29.4)</td>
</tr>
<tr>
<td>Co-morbidity</td>
<td></td>
</tr>
<tr>
<td>Obesity</td>
<td>5 (29.4)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>5 (29.4)</td>
</tr>
<tr>
<td>Type 2 DM</td>
<td>3 (17.6)</td>
</tr>
<tr>
<td>Obstructive sleep apnea</td>
<td>2 (11.8)</td>
</tr>
<tr>
<td>Allergy</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Cerebrovascular accident</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Depression on treatment</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Medically free</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>Information is not available</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Pronounced cause of death&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>7 (41.2)</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>Coronary artery thrombus</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>Multiorgan failure</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Intracranial hemorrhage</td>
<td>1 (5.9)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pronounced cause of death: before autopsy examination.

DM, diabetes mellitus.

DISCUSSION

This study represents the first description of autopsy findings from COVID-19.
### Table 2. Autopsy lung and heart findings in the study cohort (n = 17)

<table>
<thead>
<tr>
<th>Finding</th>
<th>Total cases (n = 17)</th>
<th>Died of COVID-19 (n = 13)</th>
<th>Died with SARS-CoV-2 infection (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lung</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gross</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion and edema</td>
<td>12 (70.6)</td>
<td>10 (76.9)</td>
<td>2 (50.0)</td>
</tr>
<tr>
<td>Hepatization</td>
<td>10 (58.8)</td>
<td>7 (53.8)</td>
<td>3 (75.0)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>10 (58.8)</td>
<td>9 (69.2)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Vascular thrombosis</td>
<td>3 (17.6)</td>
<td>3 (23.1)</td>
<td>0</td>
</tr>
<tr>
<td>Pleural adhesions</td>
<td>3 (17.6)</td>
<td>3 (23.1)</td>
<td>0</td>
</tr>
<tr>
<td>Saddle pulmonary embolism</td>
<td>2 (11.8)</td>
<td>2 (15.4)</td>
<td>0</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>1 (5.9)</td>
<td>1 (7.7)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Histopathologic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveolar congestion only</td>
<td>4 (23.5)</td>
<td>0</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Exudative diffuse alveolar damage</td>
<td>5 (29.4)</td>
<td>5 (38.5)</td>
<td>0</td>
</tr>
<tr>
<td>Proliferative diffuse alveolar damage</td>
<td>7 (41.2)</td>
<td>7 (53.8)</td>
<td>0</td>
</tr>
<tr>
<td>Early fibrosing diffuse alveolar damage</td>
<td>1 (5.9)</td>
<td>1 (7.7)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Specific microscopic findings</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaline membranes with type I pneumocytes necrosis or denudation</td>
<td>5 (29.4)</td>
<td>5 (38.5)</td>
<td>0</td>
</tr>
<tr>
<td>Organizing or remnants of hyaline membrane and type II pneumocytes hyperplasia</td>
<td>7 (41.2)</td>
<td>7 (53.8)</td>
<td>0</td>
</tr>
<tr>
<td>Early and focal fibrosis</td>
<td>1 (5.9)</td>
<td>1 (7.7)</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse collagenous fibrosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Interstitial and intra-alveolar edema</td>
<td>17 (100)</td>
<td>13 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Microscopic honeycomb-like change</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Collapsed alveoli</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytic infiltration</td>
<td>11 (64.7)</td>
<td>11 (84.6)</td>
<td>0</td>
</tr>
<tr>
<td>Traction bronchiectasis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Pneumocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No change</td>
<td>4 (23.5)</td>
<td>0</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Denudation and necrosis of type I pneumocytes</td>
<td>4 (23.5)</td>
<td>4 (30.8)</td>
<td>0</td>
</tr>
<tr>
<td>Proliferation of type II pneumocyte</td>
<td>3 (17.6)</td>
<td>3 (23.1)</td>
<td>0</td>
</tr>
<tr>
<td>Denudation and necrosis of type I pneumocytes and proliferation of type II pneumocyte</td>
<td>6 (35.3)</td>
<td>6 (46.2)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Necrosis of endothelial cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16 (94.1)</td>
<td>12 (92.3)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Yes</td>
<td>1 (5.9)</td>
<td>1 (7.7)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Neutrophil aggregation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16 (94.1)</td>
<td>12 (92.3)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Yes</td>
<td>1 (5.9)</td>
<td>1 (7.7)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Microthrombi</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>9 (52.9)</td>
<td>6 (46.2)</td>
<td>3 (75.0)</td>
</tr>
<tr>
<td>Present</td>
<td>8 (47.1)</td>
<td>7 (53.8)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td><strong>Hemorrhage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>5 (29.4)</td>
<td>3 (23.1)</td>
<td>2 (50.0)</td>
</tr>
<tr>
<td>Present</td>
<td>12 (70.6)</td>
<td>10 (76.9)</td>
<td>2 (50.0)</td>
</tr>
<tr>
<td><strong>Pulmonary capillary congestion</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Absent</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Present</td>
<td>17 (100)</td>
<td>13 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td><strong>Reactive pneumocytes and syncytial cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>7 (41.2)</td>
<td>4 (30.8)</td>
<td>3 (75.0)</td>
</tr>
<tr>
<td>Present</td>
<td>10 (58.8)</td>
<td>9 (69.2)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td><strong>Focal emphysema</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>9 (52.9)</td>
<td>7 (53.8)</td>
<td>2 (50.0)</td>
</tr>
<tr>
<td>Present</td>
<td>8 (47.1)</td>
<td>6 (46.2)</td>
<td>2 (50.0)</td>
</tr>
</tbody>
</table>

(Continued to the next page)
from individuals positive for COVID-19 in Jordan, and Arab countries to the best of our knowledge. Previously, a majority of autopsy case series in the context of COVID-19 were mostly conducted in the United States, Europe, and China [21]. Such a study appears of prime importance in the Middle East region for a number of reasons. First, challenges were posed to forensic pathologists considering the controversies around the Islamic burial rituals and handling of corpses if autopsies revealed the presence of SARS-CoV-2 [33,34]. This is particularly relevant considering the evidence suggesting the presence of replicative SARS-CoV-2 a few days postmortem as shown in the study by Grassi et al. [35] that involved 29 autopsies examined in Italy. Our findings confirmed the presence of the virus in all cases included in this study. The diagnosis of COVID-19 in this case series was established by postmortem molecular or serologic testing for SARS-CoV-2 even in the absence of typical clinical presentation of COVID-19 at time of death. Second, this study is important in Jordan considering the previous evidence of high prevalence of embracing conspiratorial beliefs towards emerging virus infections that could entail irrational COVID denialism [36]. The findings of this study add an explicit proof of the presence of the virus among autopsies that tested positive for the virus through direct EM visualization. The confirmation of coronavirus presence by EM examination added further proof of virus presence in all included cases despite the variability in laboratory testing approach initially used in this case series (qPCR vs. serologic testing).

Macroscopic and microscopic findings in the majority of cases (13/17, 76.5%) in this study were consistent with previous literature suggesting the crucial role of DAD as the hallmark of acute respiratory distress syndrome complicating COVID-19 infections [37-39]. The predominance of proliferative DAD in this case series indicates the late presentation of a substantial number of cases with subsequent ominous outcome. The wide prevalence of DAD as the predominant feature in lung parenchyma among COVID-19–positive autopsies was reported previously in various reports from the United States [40,41], Germany [30,42], Spain [43], Italy [44], and China [45]. An early

<table>
<thead>
<tr>
<th>Table 2. Continued</th>
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</thead>
<tbody>
<tr>
<td>Finding</td>
</tr>
<tr>
<td>Vasculitis</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Present</td>
</tr>
<tr>
<td>Heart</td>
</tr>
<tr>
<td>Gross</td>
</tr>
<tr>
<td>No significant changes</td>
</tr>
<tr>
<td>Coronary arteries atherosclerosis</td>
</tr>
<tr>
<td>Cardiac hypertrophy</td>
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<td>Cardiomegaly</td>
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<tr>
<td>Acute thrombus</td>
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<tr>
<td>Fibrosis</td>
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<tr>
<td>Specific microscopic findings</td>
</tr>
<tr>
<td>Fibrosis in intima</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Present</td>
</tr>
<tr>
<td>Thickening of media</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Present</td>
</tr>
<tr>
<td>Myocardial hypertrophy</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Present</td>
</tr>
<tr>
<td>Senile amyloidosis</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Present</td>
</tr>
<tr>
<td>Myocardial cell necrosis</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Present</td>
</tr>
</tbody>
</table>

Values are presented as number (%). COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
case series from Switzerland that involved 21 autopsies examined following COVID-19–related mortalities revealed that the major cause of death was respiratory insufficiency due to exudative DAD and massive capillary congestion [46]. In addition, the Swiss case series reported the detection of microthrombi despite the intake of anticoagulation prior to death [46].

The role of coagulopathy in the pathophysiology of COVID-19 was manifested by the frequent detection of microthrombi in lungs (8/17, 47.1%) in the study cohort. Likewise, and indicative of the central role of thrombotic microangiopathy in critical cases of COVID-19, a study from Iran reported the presence of thrombotic microangiopathy in 60% of 31 lung biopsies from patients who passed away due to COVID-19 [47]. Additional reports on autopsy findings amid COVID-19 pandemic found a high incidence of thromboembolic events suggesting its role in the fatal outcome of COVID-19 [39,48]. A recent comprehensive review for autoptic investigations from of 749 COVID-19 mortalities in 14 studies revealed the presence of pulmonary embolism-related findings in 30% of cases, with venous thromboembolic events as the cause of death 25% of the cases [49]. An early Austrian study involving 11 autopsies showed that thrombosis in small pulmonary arteries was a fundamental finding resulting in mortality due to COVID-19 [50].

The frequency of co-morbidities was high in this case series among mortalities that were linked directly to SARS-CoV-2 infection in contrast to its total absence among those who died with the disease. Conditions like hypertension, obesity, and type 2 diabetes mellitus were previously linked to excess mortality among SARS-CoV-2 infected patients [51]. In line with this observation, co-morbidities in this cohort were linked to death from COVID-19 rather than death with the virus.

A novel finding of this study was the observation that four out of 17 individuals died without significant histopathologic pulmonary changes. This observation was made despite the presence of SARS-CoV-2 as evidenced by qPCR, serology, and EM findings. One important parameter in the assessment of infec-
The proportion of infectious disease is the CFR, which is defined as “the proportion of individuals diagnosed with a disease who die from that disease” [3]. The aspects of bias in the efforts to estimate COVID-19 CFR appear to cause an underestimation through time lags in reporting of death, and overestimation through underreporting of asymptomatic and mild cases [5]. Another important cause of bias, which was noticed in this study, is the overestimation of fatalities due to COVID-19. This can happen as a result of reporting any death with a positive SARS-CoV-2 testing result as a COVID-19 case, even in the absence of sufficient evidence that the individual died as a result of virus infection, which might be present as an incidental finding [52]. Such a scenario can frequently occur in the course of COVID-19 epidemic waves with a high proportion of asymptomatic cases/mild disease [16]. Hence, subclinical cases are rarely tested and consequently are missed.

In this study, even with a small sample size, the proportion of death with SARS-CoV-2 infection appears relatively high 4/17 (23.5%). In line with this finding, a study from Italy reported on the causes of death among nine cases that tested positive for COVID-19 at time of death, with five cases dying as a result of carbon monoxide poisoning in a nursing home (death with SARS-CoV-2 infection) [11]. In an earlier study from Germany, Edler et al. [42] found a similar observation; however, at a much smaller scale with only 5% out of 80 autopsies that were labeled as “non-COVID-19 deaths.”

It is important to note that the incidental finding of SARS-CoV-2 in autopsies can be higher in outbreak situation. The current study took place during the first and second waves of COVID-19 in Jordan, with more than several thousand of newly diagnosed cases at waves’ peaks [53]. Several differential diagnoses should be considered in outbreak setting with community spread of SARS-CoV-2, including other viral infections, previously undiagnosed heart disease, and drug toxicity among other conditions [54]. Despite the absence of DAD in four cases, the role of SARS-CoV-2 in mortality cannot be ruled out, particularly in the cases with ACS and this issue could be viewed as a caveat in our stratification approach. This is evidenced by the previous studies linking COVID-19 with direct cardiac damage and indirect involvement through the thrombotic complications [55]. However, we are inclined to believe that the cases in the “death with SARS-CoV-2 infection” group did not pass away due to SARS-CoV-2 complications since the subjects in this group lacked co-morbidities and were younger compared to those with histopathologic evidence of death due to the infection.

Finally, the findings in this case series should be interpreted in light of several limitations that included: (1) the small sample size, which was mostly related to restriction of autopsy examination to medicolegal cases; (2) potential selection bias since all deaths were considered as medicolegal cases; (3) missing of full clinical history data in a few cases, including the history of COVID-19 vaccine uptake among the four cases included following the start of vaccination campaign in Jordan, besides the lack of data on microbiologic testing to rule out bacterial superinfections; (4) the approach used to stratify the cases into “died of COVID-19” vs. “died with SARS-CoV-2 infection” depended on the detection of DAD solely. Thus, future studies should benefit from a refined approach of classification including consideration of detailed medical records from the included cases, as well as the full utility of postmortem radiology, besides toxicologic investigations [20]; and finally (5) virtual autopsy (virtopsy) was not conducted in this study and should be considered in the future studies considering its promising role for postmortem investigation.

Fig. 4. The electron microscopic findings from two cases. Intracellular coronavirus particles, some of which showing delicate surface spikes (scale bar = 100 nm): ×20,000 (A), ×98,000 (B).
in the context of resolving disputed cases of COVID-19 deaths. To conclude, in this case series, we described the histopathologic findings of COVID-19–related mortalities and explored the distinction between death due to COVID-19 as opposed to dying with SARS-CoV-2 infection. This disparity might be supported by the younger age and absence of comorbidities in the “died with COVID-19” group; however, this observation is pending further evidence from studies with larger samples. Besides DAD as the primary histopathologic finding among the “death from COVID-19” group, microthrombi were frequently detected. This microthrombi can be indicative of a hypercoagulability state. Such a state appears to play a prominent role in the pathophysiology of severe and critical cases of COVID-19, which can be implicated in the mortality from the disease.

Ethics Statement
This study was approved by the Institutional Review Board of Jordan University Hospital (JUH–IRB, decision No. 78/2021, reference No. 10/2021/5885, issued on 14 March 2021). The written informed consent from the next of kin was waived based on the medicolegal status of the cases.

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.

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Conceptualization: MA, MAA. Data curation: MA, IT, MS, ABH, IMAA, BAO, SAM, HAR, ASA, MZ, MAAA. Formal analysis: MA, MS. Funding acquisition: MA. Investigation: MA, IT, MS, ABH, IMAA, BAO, SAM, HAR, ASA, MZ, MAAA. Methodology: MA, IT, MS, ABH, IMAA, BAO, SAM, HAR, ASA, MZ, MAAA. Project administration: MA. Resources: MA, IT, MS, ABH, IMAA, BAO, SAM, HAR, ASA, MZ, MAAA. Supervision: MA, MAA. Validation: MA, MS. Visualization: MA, MS. Writing—original draft preparation: MA, MS. Writing—review & editing: MA, IT, MS, ABH, IMAA, BAO, SAM, HAR, ASA, MZ, MAAA. Approval of final manuscript: all authors.

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

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Clinicopathologic significance of the delta-like ligand 4, vascular endothelial growth factor, and hypoxia-inducible factor-2α in gallbladder cancer

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Background: Gallbladder cancer (GBC) is usually detected in advanced stages with a low 5-year survival rate. Delta-like ligand 4 (DLL4), vascular endothelial growth factor (VEGF), and hypoxia-inducible factor-2alpha (HIF2α) have been studied for their role in tumorigenesis and potential for therapeutic target, and multiple clinical trials of the agents targeting them are ongoing. We investigated the expression of these markers in surgically resected GBC and tried to reveal their association with the clinicopathologic features, mutual correlation of their expression, and prognosis of the GBC patients by their expression. Methods: We constructed the tissue microarray blocks of 99 surgically resected GBC specimens and performed immunohistochemistry of DLL4, VEGF, and HIF2α. We used the quantitative digital image analysis to evaluate DLL4 and VEGF expression, while the expression of HIF2α was scored manually. Results: The expression of VEGF and HIF2α showed a significant trend with tumor differentiation (p = .028 and p = .006, respectively). We found that the high DLL4 and VEGF expression were significantly correlated with lymph node metastasis (p = .047, both). The expression of VEGF and HIF2α were significantly correlated (p < .001). The GBC patients with low HIF2α expression showed shorter recurrence-free survival than those with high HIF2α expression. Conclusions: This study suggested the possibility of the usage of DLL4 and VEGF to predict the lymph node metastasis and the possibility of VEGF and HIF2α to predict the expression level mutually. Further studies may be needed to validate our study results and eventually accelerate the introduction of the targeted therapy in GBC.

Key Words: Gallbladder neoplasms; DLL4; Vascular endothelial growth factor; HIF2α; Targeted therapy

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Gallbladder cancer (GBC) ranks in the top 10 for both incidence and mortality in South Korea [1]. GBC is known as a deadly disease because only 21.8% of the patients are diagnosed at a localized stage, while near half (41.0%) present distant metastasis at the time of diagnosis in Korea [2,3]. The recently reported 5-year survival rate of total Korean GBC patients was 28.7%, and in cases with distant metastasis, it was even lower as 2.5% [2]. The poor prognosis of Korean GBC patients suggests the limited efficacy of conventional systemic treatments such as gemcitabine plus cisplatin, 5-fluorouracil, etc. It implicates the need to develop other treatment modalities for advanced-stage patients [4]. In the era of molecular pathology, there have been many attempts to discover actionable targets in GBC, and there are several ongoing clinical trials with novel therapeutic agents including tyrosine kinase inhibitors and immunotherapeutic agents [5,6].

Delta-like ligand 4 (DLL4) is one of the transmembrane agonistic ligands of Notch receptors [7,8] which is induced by vascular endothelial growth factor (VEGF). It plays a role as a negative feedback regulator to prevent over-exuberant angiogenesis and promote the proper formation of vascular structures [9]. Furthermore, DLL4 in vasculature cells is also involved in tumor angiogenesis through interactions with the VEGF pathway [9,10]. Previous studies demonstrated that high DLL4 expression was correlated with or predicted poor prognosis in gastric cancer and pancreatic cancer patients [11,12]. In other cancer types, includ-
ing breast cancer and head and neck squamous cell carcinoma, several studies were executed regarding DLL4 as a potential therapeutic target [13-15]. In more recent years, there have been multiple clinical trials for the efficacy of anti-DLL4 antibodies and anti-DLL4/anti-VEGF bispecific antibodies in advanced solid tumors [16,17].

Hypoxia-inducible factor-2alpha (HIF2α) is a transcription factor that is stabilized in hypoxic conditions and activates multiple downstream genes, including VEGF [18,19]. Hypoxia, in turn, increases the expression of Notch ligands, including DLL4, whereas Notch signaling regulates the response for hypoxia in multiple cancers by controlling the expression of HIF2α [18]. It is well known that the VHL-HIF2α-VEGF axis is involved in tumor development and progression of conventional clear cell type renal cell carcinoma (RCC), and the treatment options that target the molecules in this pathway have progressed in recent years. The target therapies for RCC include: targeting angiogenesis through VEGF inhibitors, anti-proliferative agents targeting the mammalian target of rapamycin pathway, immune-checkpoint inhibitor, and novel HIF2α inhibitors [20,21].

Based on the accumulated data about the interactions and associations of DLL4, VEGF, and HIF2α, we assumed that it is worthy of analyzing the association between the expression of these markers in GBC. We aimed first to find out whether the expression levels are associated with the clinicopathologic characteristics and prognosis of the patients and whether there is an association between the expressions of the three markers. On the therapeutic aspect, if substitution between an anti-DLL4 antibody or anti-DLL4/anti-VEGF bispecific antibody and HIF2α-inhibitor would be proved possible in the future based on our results, it would be a benefit for patients considering possible adverse effects or resistance [16,20].

**MATERIALS AND METHODS**

**Patient selection and tissue samples**

We collected the tissues of the GBC patients that underwent surgical resection between January 2010 and December 2017 from the surgical pathology database of Samsung Medical Center (Seoul, Korea). Initially, 101 cases were found, but one was excluded because the tumor was a metastatic tumor from the liver, and another one was excluded due to pre-operative chemotherapy. Finally, we enrolled a total study population of 99 GBC cases. Clinical data, including age, sex, date of surgery, history of post-operative chemotherapy, recurrence-free survival (RFS), overall survival, and duration of follow-up, were extracted from electronic medical records. As all hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists (K.T.J. and S.P.), the histologic type and differentiation were reviewed for all tumor tissues. We checked the tumor staging according to the American Joint Committee on Cancer staging system (8th edition) [22].

**Tissue microarray construction and immunohistochemistry**

Representative tumor areas confirmed for the absence of hemorrhage or necrosis were marked on the formalin-fixed paraffin-embedded blocks. Two tissue cores with a diameter of 2.0 mm were acquired from each donor block and were arranged in the recipient paraffin blocks. Each tissue microarray (TMA) block contained up to 40 tumor tissue cores and two control tissue cores. One normal pancreas tissue core and one normal tonsil tissue core were used as control cores.

Immunohistochemistry (IHC) was performed on 4-μm-thick tissue sections obtained from TMA blocks. For detection of DLL4 and HIF2α, automated Ventana BenchMark Ultra instrument (Ventana Medical Systems, Tucson, AZ, USA) was used for antigen retrieval and primary antibody reaction. After the antigen retrieval for 92 minutes with CC1 in Ventana BenchMark Ultra, the sections were incubated with anti-DLL4 antibody (HPA023392, 1:50, Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes in 37°C and with EPAS-1 (sc-46691, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for HIF2α detection for 120 minutes in 37°C, respectively. For EPAS-1, the chromogenic reactions were carried out for 12 minutes with OptiView Amplification Kit (860-099, Ventana Medical Systems) and OptiView DAB IHC Detection kit (760-700, Ventana Medical Systems, Tucson, AZ, USA) was used for antigen detection for 120 minutes in 37°C, respectively. For EPAS-1, the chromogenic reactions were developed for 10 minutes using the Bond Polymer refine detection kit, DS9800 (Vision Biosystems, Melbourne, Australia).

For VEGF detection, the sections were incubated with a mouse monoclonal anti-VEGF antibody (sc-7269, 1:500, Santa Cruz Biotechnology) for 20 minutes in a Bond-max autoimmunostainer (Leica Biosystems). The TMA slide stained with H&E and IHC was digitized by Aperio AT2 scanner (Leica Biosystems, Buffalo Grove, IL, USA) after antigen retrieval with ER1 buffer (pH 6.0, Leica Biosystems) in 100°C. Antigen-antibody chromogenic reactions were developed for 10 minutes using the Bond Polymer refine detection kit, DS9800 (Vision Biosystems, Melbourne, Australia).

**Quantitative digital image analysis and manual scoring**

The TMA slide stained with H&E and IHC was digitized by Aperio ImageScope software (ver. 12.4.2, Leica Biosystems, Buffalo Grove, IL, USA) at 20× magnification. For DLL4 and VEGF expression analysis, Aperio ImageScope software (ver. 12.4.2, Leica Biosystems, Buffalo Grove, IL, USA) was used. All tumor cells except those
in lymphocyte-rich areas were exclusively annotated in each TMA core. According to the expression patterns of the antibodies, DLL4 expression and VEGF expression were analyzed with membrane v9 algorithm and cytoplasm v2 algorithm (Fig. 1), respectively. The algorithms were available as a component in the commercial version of Aperio ImageScope software. Both algorithms automatically counted the VEGF- or DLL4-positive cells based on their staining intensity (0, 1+, 2+, and 3+). The annotation was performed by one pathologist (S.P.), and reviewed by an additional pathologist (K.T.J.) before the automatic analysis. Two pathologists (S.P. and K.T.J.) jointly reviewed the digitally scanned slides and results of the automatic analysis to confirm its performance. The H-scores could be automatically derived from the results (cytoplasm v2), or be calculated from the values of the results (membrane v9).

Due to the extensive background staining of HIF2α compared to DLL4 and VEGF (Fig. 2), the expression of HIF2α was manually scored according to the staining intensity (0, 1+, 2+, and 3+) (Fig. 3) to calculate the H-scores, by one pathologist (S.P.). As an additional pathologist (K.T.J.) reviewed, consensus was achieved between two pathologists for any discrepancy.

Statistical analysis

As appropriate, Pearson’s chi-square test or Cochran-Armitage test was used to analyze the correlation between DLL4, VEGF, and HIF2α expression and clinicopathologic parameters. Pearson’s chi-square test and Spearman’s rank correlation test were used to analyze the correlations between the expression levels of three markers. The Kaplan-Meier survival method was used to analyze survival rates. The Cox proportional hazard regression was used to describe the effects of one or more predictors on survival time or time-to-event outcomes. In all statistical analyses, IBM SPSS ver. 27.0 for Windows (IBM Corp., Armonk, NY, USA) and R software (R Foundation for Statistical Computing, Vienna, Austria) were used.

RESULTS

Clinicopathologic features of the GBC patients

The clinicopathologic features of the GBC patients and the
The association of these features with DLL4, VEGF, and HIF2α expression are summarized in Table 1. For the chi-square test, the expression levels of the markers were divided into two groups: high when the H-score ≥ 120, low when < 120 (Fig. 1). Among the 99 patients, 40 patients (40.4%) were male, and 59 patients (59.6%) were female, and their age at the time of diagnosis ranged widely from 31 to 85 years (median age, 63 years). All tumors were primary GBCs, consist of conventional adenocarcinoma (81 cases, 81.8%), adenosquamous carcinoma (8 cases, 8.1%), neuroendocrine carcinoma (5 cases, 5.1%), mixed neuroendocrine carcinoma and adenocarcinoma (1 case, 1.0%), and undifferentiated carcinoma (3 cases, 3.0%, respectively). About half of the cases were well, moderately, or well to moderately differentiated tumors (56 cases, 56.6%). Forty cases (40.4%) had any proportion of poor differentiation, and the remaining three cases (3.0%) were classified as undifferentiated tumors. As a result of surgical resection, most cases were T3 (88 cases, 88.9%). Lymph node metastasis was present in 68 cases (68.7%), and distant metastasis was present in 11 cases (11.1%). Recurrence occurred in 53 cases (53.5%) during the follow-up period (median, 11.6 months; range, 0.7 to 126.9 months) and 36 patients (36.4%) died during the follow-up period (median, 20.0 months; range, 0.7 to 126.9 months).

The chi-square test showed that lower DLL4 expression and VEGF expression was associated with lymph node metastasis (p = .047, both). The Cochran-Amitage test revealed that there is a statistically significant linear-trend between the degree of differentiation and VEGF or HIF2α expression (p = .028 and p = .006, respectively). The test suggests strong evidence of a linearity between the expression of these markers and the tumor differentiation.

Expression of DLL4, VEGF, and HIF2α
When compared by the chi-square test as described above, DLL4 expression did not correlate with the expression of other markers. VEGF and HIF2α expression, however, was significantly correlated (p < .001), tumors with high VEGF expression would display higher expression of HIF2α, vice versa.
To further confirm the statistical significance of the correlation between expressions of the three markers, we performed an additional statistical analysis. By correlation matrix, the correlations were visualized, showing weak correlations between DLL4 vs. VEGF, and DLL4 vs. HIF2α, but a relatively strong correlation between VEGF vs. HIF2α (Fig. 4).

Considering that the H-score values of three makers are not normally distributed, we used Spearman’s ρ rank correlation coefficient to test the significances of correlations between the H-score values of three makers. Between DLL4 and VEGF, and between DLL4 and HIF2α, the Spearman’s rank correlation coefficients are 0.09 (p = .356) and 0.34 (p < .001), respectively, which is consistent with the insignificant results obtained with previous chi-square test. Between H-score values of VEGF and HIF2α, there was a statistically significant positive correlation, with correlation coefficient of 0.57 (p < .001). The Spearman’s rank correlation coefficient values between each marker are reflected in Fig. 4.

Impact of DLL4, VEGF, and HIF2α expression on the prognosis

According to the Kaplan-Meier survival analysis result, expression of DLL4 and VEGF did not affect recurrence or death. According to HIF2α expression, however, recurrence rates showed a statistically significant difference. When the cutoff for high or low expression was set as H-score = 150, patients with low HIF2α expression (n = 74) showed shorter RFS than the patients with higher HIF2α expression (n = 25) (p = .048). When the high expression group was defined as the tumor with more than 30% of 2+ and 3+ cells, patients with low HIF2α expression (n = 70), again, showed shorter RFS than those with higher HIF2α expression (n = 29) (p = .011) (Fig. 5). By performing Cox proportional
Table 1. The clinicopathologic features and association with DLL4, VEGF, and HIF2α expression

<table>
<thead>
<tr>
<th></th>
<th>DLL4 expression</th>
<th>VEGF expression</th>
<th>HIF2α expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n=56)</td>
<td>High (n=43)</td>
<td>p-value</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 60</td>
<td>53.2 (36.2)</td>
<td>45.7 (33.7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>&lt; 60</td>
<td>20 (12.5)</td>
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<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>37 (25.4)</td>
<td>24 (17.2)</td>
<td>.023</td>
</tr>
<tr>
<td>Male</td>
<td>55 (37.5)</td>
<td>62 (45.5)</td>
<td></td>
</tr>
<tr>
<td>Diagnosis</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>5 (3.3)</td>
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</tr>
<tr>
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<td>28 (20.0)</td>
<td></td>
</tr>
<tr>
<td>NEC</td>
<td>33 (22.1)</td>
<td>37 (25.4)</td>
<td></td>
</tr>
<tr>
<td>Mixed NEC and adenocarcinoma</td>
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<td>2 (1.4)</td>
<td></td>
</tr>
<tr>
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<td>1 (0.7)</td>
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<tr>
<td>Undifferentiated carcinoma</td>
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<td>1 (0.7)</td>
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<td>.006</td>
<td>.006</td>
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<tr>
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<td>24 (17.2)</td>
<td></td>
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<tr>
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<td>12 (8.8)</td>
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<tr>
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<td>3</td>
<td>49 (33.2)</td>
<td>46 (32.1)</td>
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<td>4</td>
<td>6 (4.0)</td>
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<td>.047</td>
<td>.872</td>
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<td>.772</td>
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<td>.174</td>
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<tr>
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<td>29 (19.7)</td>
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<tr>
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<td>.789</td>
<td>.549</td>
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<tr>
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<tr>
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<td>.340</td>
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<tr>
<td>High</td>
<td>16 (11.0)</td>
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</tr>
<tr>
<td>Low</td>
<td>18 (12.5)</td>
<td>18 (12.5)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>27 (18.7)</td>
<td>27 (18.7)</td>
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</tr>
</tbody>
</table>

Values are presented as number (%).

DLL4, delta-like ligand 4; VEGF, vascular endothelial growth factor; HIF2α, hypoxia-inducible factor-2α; NEC, neuroendocrine carcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; UD, undifferentiated; AJCC, American Joint Committee on Cancer.

*By Cochran-Armitage trend test, otherwise by chi-square test.

hazard regression, HIF2α expression was confirmed to be a significant predictive factor of the time to recurrence (p = .020), along with the presence of nodal metastasis of the patients (p < .001). The result regarding HIF2α expression shows consistency with the result of Kaplan-Meier survival analysis. Overall survival rate did not differ among patients according to HIF2α expression by Kaplan-Meier survival analysis, and all the negative results were consistent with the result of Cox proportional hazard regression model.
DISCUSSION

Although several previous studies investigated the expression of DLL4, VEGF, or HIF2α in GBC separately [23-27], this is the first study to perform the IHC of these three markers coincidently, attempting to integrate and confirm the results of the previous studies. As the study material, we used the TMA constructed with 99 surgically resected gallbladder cases from a single institution. These cases were followed up for a relatively long period of time – at least 0.7 months to a maximum of 11 years. For analysis of IHC, we digitized the slides and utilized a digital image analysis platform. Complete annotation of tumor area of 99 cases was laborious and time-consuming, but with this tool, we could assure the objectivity of the analysis. Most of the previous studies scored the IHC stain manually with a light microscope, which is inevitably subjective, and this might affect the study results. With digital image analysis, we attempted to overcome such limitations on the aspect of DLL4 and VEGF, but still not for the analysis related to HIF2α, due to the background staining (Fig. 2D). Quantitative digital image analysis has been used in multiple previous studies in various platforms such as Aperio ImageScope, QuPath, ASAP, etc. Since subjectivity of interpretation has long been a hurdle to be overcome and a task for pathologists to conquer, we believe this can be achieved in part by digital image analysis.

The tumors were divided into three groups according to the degree of differentiation. The tumors that consisted of only well-differentiated and/or moderately differentiated portions were clas-
sified as the first group, and the tumors with any poorly differentiated portion were the second group. Only three undifferentiated tumors were classified as the third group. As a result, there was a positive trend between the differentiation and the expression of VEGF or HIF2α; the worse the differentiation tumors showed, the lower expression they exhibited. This trend was not consistent with the results of previous studies: some studies showed that VEGF expression was higher in poorly differentiated tumors compared to more differentiated tumors [25, 27], and in other studies, no association was revealed [23, 24]. To our knowledge, there have been no previous studies to report the significant association of degree of tumor differentiation and HIF2α expression. Although this study demonstrated a different result than the previous ones because the expression of VEGF and HIF2α are positively correlated, which is consistent with what is stated in other studies. Therefore, the result regarding the trend with differentiation might not be discarded. Instead, such conflicting results should be explained by investigating underlying mechanisms in future studies.

As stated above, VEGF and HIF2α expression were significantly correlated — the tumors with higher VEGF expression tend to show higher HIF2α expression, vice versa. In a study by Giatromanolaki et al. [23], IHC was performed in 60 GBC samples and showed a similar result compared to ours. Since the data showing the association between VEGF and HIF2α expression are being accumulated, the application of these data onto the therapeutic aspects of these markers could be considered. VEGF has long been regarded as a well-established anti-neoplastic therapy. Several anti-VEGF inhibitors have been developed and are currently used, including bevacizumab [28]. Anti-VEGF inhibitor inhibits angiogenesis by reducing endothelial cell proliferation and thus tumor growth. Recently, anti-DLL4/anti-VEGF bispecific monoclonal antibody has been developed to enhance the anti-neoplastic activity and avoid the cardiac toxicity observed in patients when treated with anti-DLL4 inhibitors [16]. HIF2α, on the other hand, playing a pivotal role in tumor progression and metastasis [19], and being the main driver in the development of clear cell RCC [29], is an attractive therapeutic target. Multiple agents have been designed, and some have shown promising results in preclinical level and clinical trials [30, 31]. Since the role of both VEGF and HIF2α and drugs that inhibit their action are being vigorously investigated, if VEGF and HIF2α could work as surrogate markers for each other or helps predict the expression level of each other, it might be considerably useful and convenient, in possible future occasions that expression level of these markers may work as a treatment indication.

By chi-square test, we found that DLL4 and VEGF are correlated with lymph node metastasis status. DLL4 and VEGF expression tended to be lower in cases with lymph node metastasis (p = .047). Although our result did not reveal any prognostic significance of DLL4 and VEGF, because lymph node metastasis is determining factor for TNM stage that reflects patient survival, this correlation may point to the potential prognostic implication. In the cases that show low expression when stained with DLL4 and VEGF and if it is detected in biopsy sample prior to surgical resections, surgeons should perform meticulous lymph node dissection considering the higher possibility of lymph node metastasis. The pathologists should also spend more time evaluating the presence of tumor cells in dissected lymph nodes. If such a patient is subject to concurrent chemotherapy and radiotherapy, it would provide information for oncologists or radiologists’ decision. Low expression groups of DLL4 and VEGF, however, account for more than half of the patients (65.7% and 56.6%, respectively), there is the possibility that they might not work as the effective screening tool. A study by Liu et al. [32] showed a relevant result in non-small cell lung cancer cases, stating that low DLL4 expression was significantly correlated with lymph node metastasis. A study with a conflicting result compared to ours [33] revealed that high DLL4 expression predicted pelvic lymph node metastasis in early cervical cancer patients. Moreover, high DLL4 expression was an independent predictor of poor survival in these cervical cancer patients. Such conflict is possibly due to the bi-functional cellular responses that Notch signaling pathway may induce during tumorigenesis in different tumors [17]. DLL4, working as a ligand in the Notch signal pathway, may either promote or inhibit tumor cell proliferation or survival, and this might be different according to tumor cell origin of tumor cell types. Further studies are necessary to clarify the underlying mechanism of DLL4 activity in GBC to confirm our results on lymph node metastasis.

Except for HIF2α, two other markers did not show any correlation with prognosis. The correlation of HIF2α with recurrence was not clear when the patients were divided into two groups: high as H-score ≥ 120 or low as H-score < 120. When the high group was set with more conservative criteria (higher H-score) or the proportion of 2+ and 3+ cells, patients with lower HIF2α expression showed shorter RFS than those with higher HIF2α expression. By Cox proportional hazard regression model, HIF2α expression was confirmed as a significant predictive factor for recurrence. High HIF2α expression, therefore, may help to expect a better prognosis regarding recurrence, but the threshold for “high” expression should be relatively high to gain
In our study cohort, some of the patients were transferred to different hospitals right after surgery for subsequent treatment and follow-up. The data regarding recurrence, death, and additional treatment could be incomplete, which might have affected our results.

In this study, multiple cutoffs for statistical analysis were used. For example, H-score = 120, H-score = 150 or the tumor with more than 30% of 2+ and 3+ cells, in each analysis. In studies using quantitative measuring of expression level, especially regarding the studies using immunohistochemical stain, a certain cutoff is needed for grouping the patients. The gold standard for setting the cutoff value, however, is not established or even recommended for pathologists. H-score = 120 was helpful in dividing the patients into two groups with adequate population in each group, for all three markers (DLL4: n = 65 in low group, n = 34 in high group; VEGF: n = 56 in low group, n = 43 in high group; HIF2α: n = 65 in low group, n = 34 in high group). For survival analysis, however, with the same cutoff no statistically significant result was yielded, so that other cutoff values were adopted and utilized in this study. Considering that all researchers should report any meaningful data they obtained during the analysis, it was unavoidable to report the statistically significant results with multiple cutoffs.

In a previous study that demonstrated DLL4 expression was a prognostic marker and predicted gemcitabine effect in pancreatic cancer [34], two cohorts of patients, total 154, were enrolled and their clinicopathologic and treatment data for at least 6 years were collected. When a larger number of patients with complete data for survival and post-operative treatment is available in our study, DLL4 expression is worth being re-evaluated for its prognostic significance in association with treatment effect like the study by Drouillard et al. [34]. Our study has other limitations. The study cohort only includes surgically resected cases. Although 25 cases were advanced diseases as to be staged surgically as IVB, including 11 cases with distant metastasis and 14 cases without distant metastasis but with N2 lymph node metastasis, the majority of this cohort was relatively early GBC cases. Because GBC is one of the lately detected cancers, most of the patients are subject to systemic treatment rather than surgical resection at the time of diagnosis. Our cohort, therefore, may not represent the whole GBC patients. Another limitation is that we could not evaluate the HIF2α expression digitally. Objectivity gained by quantitative digital image analysis for DLL4 and VEGF is one of the strengths of our study. However, due to the intensive background stain of HIF2α (Fig. 2), there was no available tool to annotate and evaluate the intensity of staining exactly. Although the consensus was made between two pathologists, manual evaluation might be relatively crude and subjective compared to digitized analysis.

In conclusion, this study studied the expression patterns and levels of DLL4, VEGF, and HIF2α in surgically resected GBC. We demonstrated that VEGF and HIF2α expression intensity is positively correlated, suggesting the possibility for these markers to work as mutually substitutable markers. Low DLL4 and VEGF expression levels were significantly associated with the status of lymph node metastasis, presumably with prognosis, although such a result was not yielded in this study. Lastly, patients with lower HIF2α expression showed shorter RFS in our cohort. The cellular mechanisms of DLL4, VEGF, and HIF2α in GBC are worth further investigating to explain these results, to accelerate the application the target therapy for these molecules to treat GBC patients.

**Ethics Statement**

The institutional review board of Samsung Medical Center approved this study (2021-10-053) and waived 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.

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**Conflicts of Interest**

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

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**References**


Unsuspected systemic Epstein-Barr virus–positive T-cell lymphoma of childhood diagnosed at autopsy in a potential homicide case

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Systemic Epstein-Barr virus (EBV)-positive T-cell lymphoma of childhood (SETLC) is a rare, rapidly progressive, and often fatal disease of children and young adults characterized by monoclonal expansion of EBV-positive T cells in tissues or peripheral blood following infection with EBV. Its distinction from other EBV-positive T-cell lymphoproliferative disorders with overlapping features can be difficult, and particular diagnostic features may not be manifest until autopsy examination. We present the case of a 10-year-old boy with significant disability due to remote traumatic brain injury following non-accidental head trauma who died unexpectedly at home. Given the history of physical abuse and the potential for homicide charges, significant medicolegal implications arose with this case. Pathologic investigation ultimately revealed conclusive diagnostic features of SETLC including extensive proliferation of EBV-positive T cells in multiple organs. A natural manner of death was confirmed, thereby excluding delayed homicide related to complications of non-accidental head trauma.

Key Words: Lymphoma, T-cell; Epstein-Barr virus infections; Autopsy; Homicide

CASE REPORT

The decedent was a 10-year-old white male who at approximately 20 months of age suffered TBI, multiple skeletal fractures, and bilateral retinal hemorrhages following non-accidental head trauma. Sequelae of the TBI included severe spastic quadriplegia and cognitive impairment in addition to multiple hospital
admissions for acute hypoxemic respiratory failure and pneumonia related to aspiration and viral infection. He was discharged home from the most recent admission approximately 5 weeks prior to his death. While aspiration and parainfluenza infection were noted during this admission, no diagnostic testing for EBV was performed, nor was there any documentation of prior EBV infection in the medical record. On the day of his death, the decedent reportedly experienced dyspnea before becoming unresponsive. Despite cardiopulmonary resuscitative efforts, he was ultimately pronounced dead at his residence.

Given the unexpected nature of his death and the medicolegal connotations associated with the history of physical abuse, a forensic autopsy was performed to evaluate the cause and manner of death. Internal gross examination revealed cavitary lesions of the right temporal lobe and the left frontal lobe of the brain consistent with remote blunt head trauma. The lungs displayed diffuse consolidation and parenchymal nodularity imparting a “cobblestone” appearance. Marked, extensive pulmonary hilar and mediastinal lymphadenopathy was identified with the largest lymph node measuring 3.5 cm (Fig. 1). Additionally, the gastroesophageal junction showed prominent mucosal nodularity. No significant hepatosplenomegaly was observed.

Hematoxylin and eosin stained tissue sections were prepared for microscopic examination. A section of pulmonary hilar lymph node showed vague preservation of B-cell follicles and profound paracortical expansion. The expanded paracortex was comprised of a spectrum of lymphocytes, ranging from small forms to more atypical intermediate/large forms with irregular nuclear contours, dispersed chromatin, and prominent nucleoli. Histologic sections of the lungs revealed effacement of the pulmonary architecture by sheets and expanded nodules of lymphoid cells with morphology identical to that in the lymph node along with frequent mitotic figures. Immunohistochemistry (IHC) was applied and showed the majority of atypical lymphocytes to be CD4-positive T cells that were also positive for CD2 and CD3 and showed partial, aberrant loss of CD5 and CD7. The atypical cells were negative for CD56 and CD138. A minority of cells were positive for CD8. In-situ hybridization for EBV encoded RNA (EBER ISH) was performed and showed extensive positivity within atypical lymphoid cells in the lymph node and the lungs (Fig. 2). A section of liver revealed multiple periportal lymphoid aggregates composed predominantly of atypical T cells, while the spleen also demonstrated moderate infiltration of the red pulp by atypical T cells. A diffuse T cell infiltrate with similar morphology was also observed in the stomach disrupting the mucosal architecture. EBER ISH was positive within the atypical lymphocytes in the spleen, the liver, and the gastric mucosa. IHC performed on bone marrow showed an abundance of CD163-positive histiocytes displaying readily observable hemophagocytosis and occasional small, T-cell predominant lymphoid aggregates (Fig. 3).

The gross and microscopic features were consistent with an aggressive, EBV-positive T-cell lymphoma involving multiple organs. Given the fulminant disease onset and rapid, unexpected demise of this pediatric patient, a diagnosis of SETLC was rendered.

**DISCUSSION**

EBV-associated lymphoproliferative diseases comprise a wide spectrum of reactive and neoplastic processes that can result in the transformation and proliferation of B, T, or natural killer (NK) cells [8]. Amongst the entities affecting T cells and NK cells specifically, disease features can overlap causing difficulty in establishing a diagnosis. To achieve an accurate diagnosis, a combination of clinical and pathologic details must be considered as key differences exist between the morphologic and temporal aspects of these processes [4,7].

In our case, several aspects aligned with a diagnosis of SETLC. From a temporal standpoint, the rapid demise of the patient was
consistent with the fulminant course associated with this entity. Gross and microscopic examination revealed overt T-cell lymphoma (features previously described above) with infiltration of multiple organs, demonstrating the aggressive and systemic nature of this disease. EBER ISH was positive in the T-cell infiltrates confirming an EBV-driven etiology. Additionally, prominent hemophagocytosis was identified in the bone marrow, a finding that is often associated with SETLC [9].

Other EBV-positive T-cell and NK-cell lymphoproliferative disorders to consider along with SETLC include EBV-positive he-
mophagocytic lymphohistiocytosis (HLH), systemic chronic active EBV infection (CAEBVI), hydroa vacciniforme-like lymphoproliferative disorder, and severe mosquito bite allergy. In our case, hydroa vacciniforme-like lymphoproliferative disorder and severe mosquito bite allergy could be excluded as these are primarily cutaneous disorders without profound systemic manifestations. EBV-positive HLH can present with clinical features similar to those of SETLC including fever, splenomegaly, and pancytopenia. However, while hemophagocytosis can be seen in the bone marrow, the spleen, or the lymph nodes, the proliferation of EBV-positive T cells is relatively small. Systemic CAEBVI can show some morphologic overlap with SETLC including paracortical expansion of lymph nodes, infiltration of multiple organs by EBV-positive T cells, and occasional hemophagocytosis. However, systemic CAEBVI displays reactive, nonspecific inflammatory changes and only subtle lymphoid infiltrates without cytologic atypia, as opposed to SETLC which is marked by neoplastic features, such as prominent lymphocytic proliferation and cytologic atypia. Additionally, systemic CAEBVI follows a more prolonged and less fulminant clinical course than SETLC with infectious symptoms persisting for greater than 3 months [1,9].

Regarding immunophenotype, the neoplastic T cells in our case showed aberrant, partial loss of CD5 and CD7 expression by IHC. Such aberrant loss of pan T-cell antigens is a well-known feature of T-cell lymphomas in general and has been previously described in cases of SETLC specifically [4]. Cases of SETLC occurring after acute EBV infection generally show T cells with a cytotoxic CD8-positive immunophenotype, while those developing from systemic CAEBVI usually display T cells that are CD4-positive. Rarely, cases occur which exhibit both CD4-positive and CD8-positive EBV-infected T cells [2,10]. The majority of neoplastic T cells in our case were CD4-positive, but there was no evidence to suggest progression from previous systemic CAEBVI. Rather, it seems more likely that our case of SETLC is part of the unique minority that displays CD4-positive T cells following acute EBV infection.

Investigation of pediatric deaths can be a challenging aspect of forensic pathology and requires meticulous evaluation of all case aspects, particularly when there is a component of abuse or neglect [11]. This becomes particularly important when evaluating for delayed homicides which can result from complications of a remote injury inflicted by another individual. In such cases, it is crucial to determine not only the immediate cause of death but also the proximate cause of death, which is the origi-
nal injury without which the fatality would not have occurred. Infection is an immediate cause of death that can be associated with remote blunt force injuries and quadriplegia [12]. In this case, it was necessary to exclude bronchopneumonia as an immediate cause of death, especially given the reported history of dyspnea and recurrent aspiration. Since pathologic investigation revealed conclusive diagnostic features of SETLC, and excluded an etiology related to complications of non-accidental head trauma, the manner of death was determined to be natural rather than homicide.

In conclusion, we present the case of a 10-year-old boy with rapid and unexpected death due to SETLC that was diagnosed at autopsy. Our case is especially informative as it illustrates diagnostic features of SETLC that separate it from other EBV-positive lymphoproliferative disorders of T and NK cells. Additionally, it demonstrates the necessity of thorough forensic examination in the evaluation of potential homicide deaths. Finally, this case highlights the importance of considering aggressive, fulminant T-cell lymphomas as unexpected causes of death, particularly in patients with rapid deterioration and vague, nonspecific clinical presentation [5,6,13].

Ethics Statement
Given the forensic nature of this case, it did not qualify as human subject research per the Health Sciences Institutional Review Board at the University of Wisconsin-Madison, and prior approval was therefore not required. Appropriate consent was obtained from the referring medical examiner’s office prior to performance of the forensic autopsy.

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.

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Conflicts of Interest
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Hamartomatous polyp is defined as a polyp with disorganized growth of normal cells indigenous to the organ. Especially, Peutz-Jeghers type hamartomatous polyp represents as the arborization of smooth muscle within the lamina propria beneath pre-existing normal epithelium of the organ it involves. This polyp is known to be associated with Peutz-Jeghers syndrome, which shows characteristic multiple hamartomatous polyp involvement in the gastrointestinal tract, combined with mucocutaneous symptom, familial history of Peutz-Jeghers syndrome or STK11/LTB1 mutation. However, some cases showing histologic appearance of the polyps discovered in Peutz-Jeghers syndrome while lacking other diagnostic criteria of the syndrome have been reported, and these are called solitary Peutz-Jeghers type polyps. Herein, we report a case of solitary Peutz-Jeghers type polyp covered with heterotopic epithelium. The patient was 47-year-old female without any mucocutaneous symptoms nor familial history of Peutz-Jeghers syndrome. Microscopic examination revealed Peutz-Jeghers type hamartomatous polyp in duodenum covered with gastric type foveolar epithelium. Considering the definition of hamartomatous polyp, which is, the abnormal overgrowth of the indigenous epithelial component, the histological feature of current case is noteworthy in a point that it shows proliferation of heterotopic component, rather than the indigenous component.

**Key Words:** Hamartoma; Intestinal polyps; Duodenum; Gastric mucosa; Peutz-Jeghers syndrome

CASE REPORT

A 47-year-old woman visited outpatient clinic for incidentally discovered duodenal polyp during regular medical check-up. She did not experience any associated symptoms. She had a history of invasive ductal carcinoma of both breasts in her early to mid-30s and been treated for papillary thyroid carcinoma in her late 30s.

On initial esophagogastroduodenoscopy, a pedunculated polyp occupying half of the luminal space was found in the 2nd portion of duodenum. Histological examination of a biopsy specimen mainly showed hyperplastic gastric foveolar epithelium. Following computed tomography scan revealed a 2.9-cm-sized polypoid lesion in duodenum without any evidence of metastasis (Fig. 1A). The polyp was entirely resected by endoscopic submucosal dissection (Fig. 1B). The patient was discharged without any post-procedural complications.

Histological examination of the resected polyp showed disorganized overgrowth of epithelial cells supported by thick, branching...
bundles of smooth muscles. The epithelium mainly consisted of foveolar type cells lying over mucinous glands resembling pyloric glands without dysplastic change (Fig. 2A, B). A small amount of surrounding duodenal tissue was identified at the margin of the specimen and it showed focal foveolar type epithelium among normal duodenal villous structures, suggestive of gastric metaplasia. Immunohistochemical staining for MUC5AC and MUC6 confirmed the covering hyperplastic epithelium to be gastric foveolar type with pyloric glands underneath (Fig. 3A, B).

Follow-up endoscopy after 6 months from the procedure revealed no additional polyps. In an in-depth interview afterwards, the patient insisted she had never been diagnosed with hamartomatous polyp before and denied of any familial history nor any mucocutaneous symptoms. Genetic assessment for STK11/LTB1 mutation was not done. According to these findings, the polyp was finally diagnosed as solitary Peutz-Jeghers type polyp with gastric foveolar epithelium.

**DISCUSSION**

Hamartomatous polyp is characterized by the arborization of smooth muscle bundle up to lamina propria with near-normal

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**Fig. 1.** Radiologic and endoscopic findings of the duodenal polyp. (A) Abdominal computed tomography image showing 2.9-cm-sized mass in duodenal 2nd portion. (B) A pedunculated polyp occupying half of the duodenal lumen discovered in endoscopic examination.

**Fig. 2.** Histological examination of the resected polyp. (A) Hematoxylin and eosin staining of the resected polyp showing arborizing smooth muscle bundles with overgrowth of superficial foveolar epithelium. (B) Foveolar type epithelium lying over pyloric type-like mucinous glands without dysplasia.
overlying epithelium. The surface epithelium of hamartomatous polyp is known to be identical with those of adjacent normal mucosa. Multiple hamartomatous polyps are frequently associated with various genetic syndromes, such as juvenile polyposis syndrome, Peutz-Jeghers syndrome and Cowden syndrome [1].

Of note, multiple GI tract polyps, mucocutaneous symptoms, STK11/LTB1 mutation and familial history of Peutz-Jeghers syndrome are characteristic of Peutz-Jeghers syndrome. As this syndrome represents an increased risk of developing cancers, patient with solitary Peutz-Jeghers type hamartomatous polyp must be meticulously examined to exclude a possibility of syndromic involvement. If the polyp is proved to have no association with Peutz-Jeghers syndrome, it is defined as solitary Peutz-Jeghers type hamartomatous polyp. Although the histological aspect of solitary Peutz-Jeghers type hamartomatous polyp is indistinguishable from the polyps of Peutz-Jeghers syndrome, this solitary polyp seems to be of separated entity, as it does not show STK11/LTB1 mutation characteristic of the syndrome. Furthermore, the frequently involved site is quite different. The polyp of the syndrome mostly occurs in small intestine, whereas the solitary counterpart develops mostly in sigmoid colon, followed by duodenum, rectum, jejunum, and stomach, according to the previously published literatures [2,3].

Solitary Peutz-Jeghers type hamartomatous polyp itself can provoke GI bleeding and intussusception, which may manifest as acute GI symptoms in some patients [4-6]. Some cases represented with dysplasia and even carcinogenesis [7,8]. Thus, complete resection of the polyp by either endoscopic or surgical procedure should be considered.

In our case, the patient was asymptomatic, and the polyp was found during routine health check-up. The resected polyp showed hamartomatous proliferation of gastric foveolar epithelium over pyloric gland-like structures in the duodenum. Whether this aspect is related to prior gastric metaplasia or abnormal differentiation of endoderm is unclear at this moment. To date, a case of pyloric metaplasia involving multiple hamartomatous jejunal polyps in a patient with Peutz-Jeghers syndrome and a case of solitary Peutz-Jeghers type polyp with gastric antral and fundic gland mucosa have been reported [6,9]. Considering the definition of hamartomatous polyp, which is the abnormal overgrowth of the indigenous epithelial component, the histological feature of current case is noteworthy in a point that it shows proliferation of heterotopic component, rather than the indigenous component.

**Ethics Statement**

Formal written informed consent was not required with a waiver by the appropriate IRB (Asan Medical Center IRB No. 2022-1339).

**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.

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Conflicts of Interest
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References
Unusual biclonal IgA plasma cell myeloma with aberrant expression of high-risk immunophenotypes: first report of a new diagnostic and clinical challenge

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IgA plasma cell myeloma (PCM) has been linked to molecular abnormalities that confer a higher risk for adverse patient outcomes. However, since IgA PCM only accounts for approximately 20% of all PCM, there are very few reports on high-risk IgA PCM. Moreover, no such reports are found on the more infrequent biclonal IgA PCM. Hence, we present a 65-year-old Puerto Rican female with acute abdominal pain, concomitant hypercalcemia, and acute renal failure. Protein electrophoresis with immunofixation found high IgA levels and detected a biclonal IgA gammopathy with kappa specificity. Histomorphologically, bone marrow showed numerous abnormal plasma cells (32%) replacing over 50% of the marrow stroma. Immunophenotyping analysis detected CD45-negative plasma cells aberrantly expressing CD33, CD43, OCT-2, and c-MYC. Chromosomal analysis revealed multiple abnormalities including the gain of chromosome 1q. Thus, we report on an unusual biclonal IgA PCM and the importance of timely diagnosing aggressive plasma cell neoplasms.

Key Words: Biclonal IgA; Plasma cell myeloma; Abdominal pain; High-risk phenotypes; Prognosis

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CASE REPORT

A 65-year-old female, Hispanic (from Puerto Rico) presented to the Emergency Department with a history of abdominal pain of 6 hours’ duration, a day after receiving the second dose of the coronavirus disease 2019 (COVID-19) vaccine. The patient denied fever, chills, dizziness, chest pain, trauma, and changes in urine or stool. Her past medical history included: obesity, hypertension, adult-onset diabetes, and status post cholecystectomy, but no history of smoking, alcohol intake, or of COVID-19 infection. She had a family history of diabetes and hypertension. Physical examination revealed mild tachycardia but was otherwise unremarkable. Subsequently, she was transferred and admitted to the hospital due to laboratory tests showing hypercalcemia with calcium level greater than 15.0 mg/dL (reference range, 8.3 to 10.6 mg/dL), increased blood urea nitrogen 36 mg/dL (refer-
ence range, 9 to 23 mg/dL) and creatinine levels 2.40 mg/dL (reference range, 0.6 to 1.1 mg/dL). Additionally, initial complete blood count results detected leukocytosis of 19.7 × 10³ cells/μL (4.3–10.3 × 10³ cells/μL) with an increase in the absolute counts detected by QxMD.

During her hospitalization, peripheral blood evaluations showed resolving leukocytosis, but worsening normocytic anemia with hemoglobin levels dropping from an initial 10.8 to 7.8 g/dL (11.3–14.8 g/dL) that required blood transfusions. Also, rouleaux formation and slight leukoerythroblastosis were noted. Serum protein electrophoresis with immunofixation (PEP/IFx) found significantly high IgA levels at 3,410 mg/dL (87–352 mg/dL) and detected two M-protein bands in the beta region confirming the presence of a biclonal IgA gammopathy with kappa specificity (Table 1). Serum levels of IgG and IgM were decreased. Urine PEP/IFx detected increased Ig in the IgA region without a well-defined band. Additionally, serum beta-2 microglobulin was markedly increased at 8.4 mg/L (0.6–2.4 mg/L).

Imaging studies including computed tomography scans of the abdomen, pelvis, and brain were done and did not find abdominal or extraosseous mass lesions. However, a whole-body bone scan identified several nonspecific lesions suspicious of metastatic infiltration or post-trauma involving the left sacroiliac joint, sternum, and the right knee lateral femoral component.

Subsequently, a bone marrow sampling was obtained from her right iliac crest. Morphological and histological evaluations of bone marrow aspirate and biopsy samples showed the following: hypercellularity (85%–95% cellularity); increase proportion of abnormal plasma cells (32%), predominantly of intermediate-type morphology with features between immature and mature plasma cells; and replacement of over 50% of the marrow stroma by the neoplastic infiltrate (Fig. 1A, B). Additionally, using reticulin and collagen stains, the presence of myelofibrosis and osteosclerosis was identified within the focal areas replaced by sheets of plasma cells and graded as MF2 of 3 [5]. No myelofibrosis was observed in the uninvolved marrow areas. Semiquantitative morphometric analysis using immunohistochemical stains were performed and revealed that a significant proportion of the marrow plasma cells expressed CD138 (> 90%) and MUM1 (> 95%) with a high kappa/lambda ratio of ~49.5. Additionally, CD43 (> 95%, a T-cell marker), OCT-2 (70%–80%), and c-MYC (15%–25%, low expression) were aberrantly expressed (Fig. 2A–D). These plasma cells did not appear to significantly express (< 20% positivity) CD10, CD56, PAX5, SOX-10, cyclin D1/BLCL1, Epstein-Barr virus, or myeloid markers (CD11c, CD15, and myeloperoxidase). However, flow cytometry studies detected CD45-negative, CD19-positive plasma cells aberrantly expressing CD33, a myeloid marker (Fig. 3A, B). Cytogenetics studies showed an abnormal complex karyotype including odd-number trisomies, and gain of chromosome 1q (Fig. 4). Then, a final diagnosis was rendered of biclonal IgA PCM with aberrant expression of multiple high-risk markers such as CD33, CD43, OCT-2, c-MYC, and gain of chromosome 1q.

Ultimately, the revised International Staging System (R-ISS) was used to estimate the patient’s prognosis [4]. Based on the Cytogenetics and other key laboratory results, the patient’s predicted R-ISS score placed her in the stage III (of III) category. This information confirming the presence of an aggressive biclonal IgA myeloma was also reported and discussed with her treating physician to help guide treatment options. However, upon being discharged, the patient was lost to follow-up.

**DISCUSSION**

Studies of newly-diagnosed patients with biclonal IgA PCM are uncommon. A possible explanation for this apparent under-reporting could be that IgA PCM is a rare entity and difficult to diagnose [6,7]. Until recently, no major differences in clinical...
presentation, prognosis, and treatment of plasma cell neoplasms were identified among patients diagnosed with IgA or the most common IgG isotype, whether expressing monoclonal or biclonal M-protein(s) [8]. However, PCM is now recognized as a genetically heterogenous neoplasm with a subset of patients targeted to develop high-risk disease [9]. Moreover, Habermehl et al. [1] reported on the poorer long-term survival of a cohort of patients with IgA gammopathy when compared to IgG patients. This recently recognized higher risk for adverse outcomes is potentially due to a decreased genomic stability in IgA neoplasms; and favors that IgA be considered in a separate diagnostic category from other PCMs. Further, their study demonstrates the importance of reporting unusual features, biomarkers, molecular abnormalities, and prognostic factors to further characterize high-risk, aggressive IgA PCM. To the best of our knowledge, this is the first report of a biclonal IgA PCM with an unusual clinical presentation in which a combination of multiple high-risk molecular phenotypes is detected.

Clinically, PCM presenting with abdominal pain as the initial symptom, like in our patient, is unusual and seldom reported. Upon review of the biomedical literature, we found only four reports of patients with IgA PCM presenting with abdominal pain (Table 2) [10-13]. In all four reports, a single monoclonal IgA paraprotein was identified. Interestingly, three of these four
cases of IgA PCM presenting with abdominal pain occurred in adults of a younger age (39, 49, 57 years) [10-12] than the reported average age for IgA patients (66.5 years) [1]. Our 65-year-old patient would be the first report of an aggressive biclonal IgA PCM presenting as new-onset abdominal pain. Hence, findings such as non-localized abdominal pain in the adult population should alert clinicians to evaluate their patients for unsuspected malignancy and should prompt the ordering of imaging studies that could lead to an early diagnosis of PCM presenting with atypical symptoms.

Histologically, the bone marrow findings together with the patient’s clinical history and key laboratory results, including biclonal IgA gammopathy, were most consistent with bone marrow involvement by an IgA PCM, following the diagnostic guidelines of the International Myeloma Working Group [2,3]. In addition, a kappa/lambda ratio of ~50 supported the presence of PCM in the patient. Moreover, since over 50% of the marrow stroma was replaced by sheets of clonal plasma cells in the bone marrow biopsy, this finding met the criteria of histologic Stage III of III, as per the proposed Histopathology-based staging system [2]. According to McKenna et al. [2], there appears to be a good correlation between the histologic stage, clinical stage, and prognosis. Therefore, these histologic findings predicted an aggressive, high-risk IgA PCM in the patient.

Immunophenotypically, the patient’s clonal plasma cells aberrantly expressed CD33 (a myeloid marker) and CD43 (a T-cell marker), and overexpressed transcription factors OCT-2 and MYC. The detection of any of these abnormal immunophenotypes in PCM is now associated with high-risk disease and poor survival [14-17]. Moreover, as published by Kodali et al. [17], MYC protein expression may serve as a potential predictor for prognosis and to assess residual disease in PCM. Based on their criteria, our patient fits in the low-MYC-expressing group (MYC < 30%) and could benefit from targeted treatment for high-risk myeloma.

Importantly, molecular cytogenetic studies in the patient identified several chromosomal abnormalities of known prognostic value in PCM. Based on the reported copy number abnormalities, her PCM could be categorized as non-hyperdiploid or hypodiploid (45 chromosomes) of complex karyotype including the loss of chromosomes14, odd-number trisomies (chromosomes

Fig. 3. Flow cytometry analysis of bone marrow cells detects increased clonal plasma cells (~11% of the viable cells; in black) that are positive for CD138 (A), lack CD45, and aberrantly express CD33 (a myeloid marker associated with high-risk myeloma) (B).

Fig. 4. Chromosome analysis revealed an abnormal complex karyotype including gain of 1q (arrow), a high-risk prognostic marker in plasma cell myeloma.
prove medical care including the use of targeted therapies, which
were still likely to die from PCM.

Although PCM survival has improved due to new treat-
ments, the majority (~72%) of Puerto Ricans diag-
nosed with PCM are still likely to die from PCM.

The translational relevance of identifying these high-risk IgA
PCMs could result in a further decline in PCM-specific mortality in
Puerto Rico.

Ethics Statement
Formal written informed consent was not required with a waiver granted
by the Institutional Review Board of Ponce Health Sciences University (IRB
No. 2110075611).

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

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Code Availability
Not applicable.

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CAMR, CNFC. Writing—review & editing: CAMR, CNFC.

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

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr), sex</th>
<th>Clinical feature</th>
<th>Ig specificity</th>
<th>M-protein clonality</th>
<th>Location</th>
<th>Microscopic findings</th>
<th>Immunophenotyping/Cytogenetics/Molecular studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annibali et al. (2009) [10]</td>
<td>39, female</td>
<td>Abdominal pain, and obstructive jaundice 7 yr after ASCT Extramedullary relapse</td>
<td>IgA-lambda</td>
<td>Monoclonal</td>
<td>Head of the pancreas, pleural effusion</td>
<td>US-guided FNA cytology of the pancreatic mass and cytology of pleural effusion revealed myeloma plasma cells</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cerqueira et al. (2020) [11]</td>
<td>49, female</td>
<td>Presented with abdominal pain, biliary vomiting of 6 days duration Admitted to ICU for acute kidney failure Extramedullary involvement</td>
<td>IgA-kappa</td>
<td>Monoclonal</td>
<td>Kidney, bone marrow</td>
<td>Kidney biopsy demonstrating myeloma kidney</td>
<td>Immunophenotyping: 60% of bone marrow monoclonal plasma cells with 100% CD38+, 100% CD33+, and 45% CD20+ Plasmacytoid cells showed CD38+, kappa+, lambda– Cytogenetics and FISH suggestive of advanced disease progression</td>
</tr>
<tr>
<td>Suo et al. (2020) [12]</td>
<td>57, male</td>
<td>History of liver cirrhosis presenting with abdominal pain and pancytopenia Extramedullary involvement</td>
<td>IgA-kappa</td>
<td>Monoclonal</td>
<td>Liver, MRI- left hepatic mass</td>
<td>Abundant plasmacytoid cells, kappa restricted neoplastic plasma cells</td>
<td></td>
</tr>
<tr>
<td>Yamane et al. (2021) [13]</td>
<td>73, male</td>
<td>Acute left lower abdominal pain</td>
<td>IgA-type</td>
<td>Monoclonal</td>
<td>Left vertebral arch of the 10th thoracic vertebra</td>
<td>Bone marrow biopsy: plasma cell neoplasm with 26.0% of plasma cells</td>
<td>Flow cytometry: CD38+, CD66+, CD138+, MPC-1+. Chromosomal analysis: 45,X,-Y,+5,+6,+7,-8,+9,+11,-13,+21</td>
</tr>
<tr>
<td>Current case</td>
<td>65, female</td>
<td>Acute abdominal pain</td>
<td>IgA-kappa</td>
<td>Biclonal</td>
<td>Bone marrow</td>
<td>Hypercellularity (85%–95%), abnormal plasma cells (32%)</td>
<td>Immunophenotyping: CD138+, CD33+, MUM-1+, CD43+, OCT-2+, c-MYC+ Chromosomal analysis: gain of 1q,13,17, loss of 14</td>
</tr>
</tbody>
</table>

ASCT, autologous stem cell transplant; US, ultrasound; FNA, fine-needle aspiration; ICU, intensive care unit; FISH, fluorescence in situ hybridization.

13 and 17), and gain of 1q [9]. These results highlight the degree of
genomic instability in her neoplastic plasma cells. According to
cardona-Benavides et al. [9], PCM patients with hypodiplo-
diploid karyotypes and those with gain of 1q tend to have more
aggressive clinical presentations and worse outcomes. Further,
the R-ISS was used to estimate the patient’s prognosis. Her pre-
dicted R-ISS score placed her in Stage III of III category with a
median overall survival of 43 months and a median progression-
free survival of 29 months [4]. Therapeutic options for these
newly-diagnosed R-ISS/Stage III PCM patients with gain of 1q
chromosomal abnormality are limited [9].

The translational relevance of identifying these high-risk IgA
PCMs could be significant for PCM patients, especially in Pu-
erto Rico. Although PCM survival has improved due to new treat-
ment modalities, the majority (~72%) of Puerto Ricans diag-
nosed with PCM are still likely to die from PCM [18]. Also, for
unknown reasons, the decline in PCM-specific mortality in
Puerto Rico is less than in other comparable US populations [18].
Future studies are needed to identify additional PCM patients
with potential high-risk phenotypes such as biclonality, IgA
subtype, MYC overexpression, gain of chromosome 1q. These
studies should establish a definitive association between these
high-risk markers and aggressive PCM. This could lead to im-
prove medical care including the use of targeted therapies, which
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