Interstitial Lung Diseases
CONTENTS

REVIEW

1  Diagnosis of interstitial lung diseases: from Averill A. Liebow to artificial intelligence
   Eunhee S. Yi, Paul Wawryko, Jay H. Ryu

ORIGINAL ARTICLES

12  Tumor-infiltrating T lymphocytes evaluated using digital image analysis predict the prognosis of patients with diffuse large B-cell lymphoma
    Yunjoo Cho, Jyeon Lee, Bogeong Han, Sang Eun Yoon, Seok Jin Kim, Won Seog Kim, Junhun Cho

22  Identification of invasive subpopulations using spatial transcriptome analysis in thyroid follicular tumors
    Ayana Suzuki, Satoshi Nojima, Shinichiro Tahara, Daisuke Motooka, Masaharu Kohara, Daisuke Okuzaki, Mitsuyoshi Hirokawa, Eichi Morii

29  Immunohistochemical expression of anaplastic lymphoma kinase in neuroblastoma and its relations with some clinical and histopathological features
    Thu Dang Anh Phan, Thao Quyen Nguyen, Nhi Thuy To, Thien Ly Thanh, Dat Quoc Ngo

CASE REPORT

35  Primary leiomyosarcoma of the bone: a case report
    Ala Abu-Dayeh, Samir Alhyassat

LETTERS TO THE EDITOR

40  Comment on “A stepwise approach to fine needle aspiration cytology of lymph nodes”
    Elisabetta Maffei, Valeria Gilberti, Pio Zeppa, Alessandro Caputo

43  Response to comment on “A stepwise approach to fine needle aspiration cytology of lymph nodes”
    Yosep Chong, Gyeongsin Park, Hee Jeong Cha, Hyun-Jung Kim, Chang Suk Kang, Jamshid Abdul-Ghafar, Seung-Sook Lee

NEWSLETTER

45  What’s new in genitourinary pathology 2023: WHO 5th edition updates for urinary tract, prostate, testis, and penis
    Bonnie Choy, Maria Tsetsiakova, Debra L. Zynger
Liebow and Carrington [1] introduced the diagnosis of usual interstitial pneumonia (UIP) over 50 years ago as a type of interstitial pneumonias. The word “usual” in UIP was used because it was the most common and usual type among interstitial pneumonias. In addition to UIP, desquamative interstitial pneumonia (DIP), bronchiolitis obliterans with interstitial pneumonia (BIP), lymphoid interstitial pneumonia (LIP), and giant cell interstitial pneumonia (GIP) were included in their classification of interstitial pneumonias [1]. While UIP, DIP, and LIP are still acknowledged in the current classification of interstitial pneumonias, BIP and GIP have been excluded; a subset of BIP might correspond to organizing pneumonia (OP) and GIP is now regarded as hard metal pneumoconiosis. Characteristic histopathologic features of various types of interstitial lung diseases (ILDs) are illustrated in Fig. 1A–D. Nearly three decades after the term UIP was first introduced, Katzenstein and Myers [2] clarified cardinal histologic features of UIP that became the current histopathologic criteria for diagnosing UIP.

The 2002 American Thoracic Society (ATS)/European Respiratory Society (ERS) statement summarized the consensus from the first international meeting on the diagnosis and treatment of idiopathic interstitial pneumonias (IIPs) [3,4]. This statement established a basic framework of the contemporary classification scheme of IIPs: UIP, nonspecific interstitial pneumonia (NSIP), DIP, OP, acute interstitial pneumonia (AIP), and LIP [4]. This nomenclature for IIP became applicable to other ILDs as patterns,
which facilitated a clearer communication in the non-idiopathic ILDs with underlying conditions or identifiable causes (such as connective tissue disease [CTD] and hypersensitivity pneumonitis [HP] or other exposure–related conditions, etc.). Some significant modifications have been made over the ensuing years in a few subsequent consensus statements by ATS and other international organizations [5–7]. Novel technologies including omics at different levels (genetic, epigenetic, transcriptional, translational, and metabolic, etc.) advanced the deep understanding in pathogenesis of ILDs and expedited the development of several effective antifibrotic medications that have been U.S. Food and Drug Administration–approved or on active clinical trials.

Pathologic examination has been the gold standard to classify ILDs until high-resolution computed tomography (HRCT) became an acceptable method after the second ATS/ERS statement in 2013, which led to a significant decrease in the frequency of lung biopsies for diagnosing ILDs [5]. Currently, most cases characteristic for UIP on chest HRCT no longer undergo lung biopsies (Fig. 2). Only atypical and complex ones are subject to lung biopsies, posing great challenges to pathologists. Surgical lung biopsy has been the main approach but transbronchial lung cryobiopsy was accepted in the 2022 practice guidelines by ATS/ERS/Japanese Respiratory Society (JRS)/Latin American Thoracic Association (ALAT) as a conditional alternative in the centers with appropriate expertise [7]. Genetic profiling of transbronchial biopsy specimens has been introduced to classify ILDs and it completely bypasses morphologic evaluation [8,9]. No recommendation was made in 2022 guidelines either for or against this

Fig. 1. (A) Usual interstitial pneumonia (UIP), the prototype of interstitial lung disease, showing patchy interstitial fibrosis with subpleural accentuation, marked architectural distortion of alveolar architecture with scarring and microscopic honeycomb changes, and temporal heterogeneity of fibrosis evidenced by scattered fibroblastic foci with dome-shaped myofibroblastic/fibroblastic proliferation over scarred areas (arrows), which likely implies an ongoing acute lung injury in already scarred lung tissue causing progressive clinical course. (B) Nonspecific interstitial pneumonia (NSIP) shows diffuse and uniform fibrous interstitial thickening. In contrast to UIP, NSIP shows diffuse collection of pigmented macrophages in the alveolar spaces with mild to moderate interstitial fibrosis. NSIP is included in the current idiopathic interstitial pneumonia classification. (C) Desquamative interstitial pneumonia (DIP) characterized by diffuse collection of pigmented macrophages in the alveolar spaces with mild to moderate interstitial fibrosis. DIP in the original classification by Dr. Liebow is included in the current American Thoracic Society classification. (D) Giant cell interstitial pneumonia (GIP) showing many scattered multinucleated giant cells in the alveolar spaces or septa. GIP was included in the Dr. Liebow’s original classification of interstitial lung diseases but dropped in the current idiopathic interstitial pneumonia classification as GIP is now primarily regarded as a hard metal pneumoconiosis (e.g., cobalt).
Recent advances in whole slide imaging (WSI) and artificial intelligence (AI) technology, such as deep learning (DL)–based image processing, have opened the door to quantitatively evaluate histopathologic findings. However, relative paucity of database, as compared to radiologic counterpart, is a limiting factor for widespread investigation and advancement to a higher level of interpretation beyond quantitative analysis, thus far.

Several topics that represent milestones in the field of ILD diagnosis are reviewed with emphasis on significant recent changes in concepts and trends relevant to practicing pathologists and pulmonologists.

**SELECTED TOPICS**

**Usual interstitial pneumonia**

**Evolution of histopathologic criteria for UIP**

The histologic classification of interstitial pneumonias evolved in a time when surgical lung biopsies were infrequently performed owing to significant morbidity associated with the surgical techniques of the day. As a result, histologic findings did not inform clinical decision-making in most patients with interstitial pneumonia. Clinicians were left to diagnose and manage patients with ILD primarily based on clinical and radiologic features; clinical concepts did not evolve in concert with the histologic classification. In this background, idiopathic pulmonary fibrosis (IPF) originated as a clinical diagnosis without established histopathologic correlates.

The original histologic description of UIP contained a wide spectrum of findings including acute lung injury in the form of what today would be considered diffuse alveolar damage, as well as chronic fibrosis and end-stage lung [2]. Although the histologic findings considered diagnostic of UIP were refined over time, established diagnostic criteria were still lacking into the 1990s with many investigators simply requiring the presence of inflammation and fibrosis in various proportions as diagnostic of UIP [3]. Many also considered even DIP to represent an early “cellular stage” of UIP [2].

In 1998, Katzenstein and Myers [2] described the crucial histologic features that were reflected in the ATS/ERS international consensus statement and established as the current histopathologic criteria of UIP [2,4]. The most recent 2022 ATS/ERS/JRS/ALAT clinical practice guideline used the same criteria including (1) patchy, dense fibrosis with architectural distortion causing destructive scarring and/or honeycombing, (2) predominantly subpleural and/or paraseptal distribution of fibrosis, (3) temporal heterogeneity of fibrosis characterized by fibroblast foci over scarred lung tissue, and (4) absence of features suggesting an alternate diagnosis (including prominent airway-centered changes, significant degree of OP, granulomas, hyaline membranes, dense lymphoid infiltrates, and marked chronic pleuritis, etc.) [4]. Different levels of confidence such as diagnostic of UIP (meeting all criteria) and probable UIP (having only some of these features) were proposed for pathologic diagnosis categories as in radiologic counterparts. This type of categorization may be useful in the clinical trial setting to recruit patients although it has not been widely applied in routine pathology practice.

In the 2013 ATS/ERS statement, the major IIPs were subdivided into three categories: chronic fibrosing interstitial pneumonias (IPF and NSIP), smoking-related interstitial pneumonias (respiratory bronchiolitis-ILD and DIP), and acute/subacute interstitial pneumonias (cryptogenic OP and AIP) [5]. The classification also includes rare IIPs (idiopathic LIP and idiopathic pleuroparenchymal fibroelastosis) and unclassifiable IIP. Each of the IIPs has an associated radiologic and/or pathologic-morphologic pattern (e.g., UIP for IPF) and is considered a “clinical-radiologic-pathologic diagnosis,” emphasizing the multidisciplinary approach to diagnosis.

**Recent trends of decreasing lung biopsy use**

The role of surgical lung biopsy in the diagnosis of IPF has changed over time. With the original ATS statement on IPF,
surgical lung biopsy was recommended in most patients to establish the diagnosis, especially in those with atypical clinical or radiologic features for IPF [4]. The 2002 ATS/ERS IIP guidelines stated that biopsy was required for a confident diagnosis of UIP/IPF, but also noted that in more than 50% of cases of suspected IPF, the presence of typical clinical and HRCT features of UIP was sufficiently characteristic to allow a confident diagnosis without the need for surgical lung biopsy [4]. In the 2011 ATS statement of IPF, radiologic and pathologic criteria were developed to establish confidence categories for UIP (i.e. UIP pattern, probable UIP pattern, possible UIP pattern); thus, surgical lung biopsy was no longer required for histopathologic confirmation in patients with HRCT showing typical UIP pattern [6]. The only change regarding sampling since that time was the adoption of transbronchial lung cryobiopsy as an acceptable alternative to surgical lung biopsy in patients with ILD of undetermined type based on evidence that there was often diagnostic agreement between transbronchial cryobiopsy and surgical lung biopsy samples [7].

**Antifibrotic therapy for patients with IPF and those with UIP due to underlying cause**

Patients with IPF undergo a progressive decline in pulmonary function with eventual death from either respiratory failure or a complicating comorbidity, with a median survival of 2 to 3 years from the time of diagnosis [6]. Acute exacerbation may occur with histologic manifestations of diffuse alveolar damage or OP, though less common [10]. Based on the hypothesis that inflammation might be the underlying cause of lung injury and fibrosis, corticosteroids and other immunosuppressive agents have been tried without success. No effective pharmacologic therapy for IPF was available until the early 2010s, when two promising antifibrotic agents emerged. Randomized trials showed in disease progression and rate of forced vital capacity decline in IPF patients treated with nintedanib, a tyrosine kinase inhibitor, or pirfenidone, an inhibitor of transforming growth factor β-associated collagen synthesis [11-14]. These two medications were recommended for use in IPF patients as of the 2015 treatment guidelines [15]. Data from more recent clinical trials led to an expansion of antifibrotic therapy to other types of fibrosing ILD if they show evidence of clinical, physiologic, or radiologic progression [4].

As such, diagnostic paradigm seems to have shifted to categorize the ILDs into two groups: cases to be treated vs. not to be treated with antifibrotics, a transition from the previous paradigm: to give or not to give corticosteroids (or other immunosuppressants). The recent proposal advocating UIP as a stand-alone diagnostic entity regardless of underlying causes is at least partly based on this therapeutic paradigm. However, such a lumping of various fibrosing ILDs might not be entirely justified, given the limited efficacy, significant side effects, and high cost of currently available antifibrotics as well as the risk of losing potentially useful diagnostic granularity. This proposal is reviewed in the section below. Similarly, the recent concept of progressive pulmonary fibrosis (PPF) published in the 2022 ATS guidelines would be in keeping with such a trend of lumping ILD diagnoses (see Concept of PPF: green light for lumping fibrosing ILD diagnoses, as exactly shown in the manuscript).

**Recent proposal of UIP as stand-alone entity**

UIP is generally regarded as the correlate of IPF. Accordingly, the cases with histopathologic features of other diseases such as fibrotic HP and CTD-related ILD are considered not consistent with UIP [16]. It is not uncommon, however, to have some minor changes associated with HP, CTD-related ILD, or other diseases (e.g., a few poorly formed granulomas, interstitial chronic inflammatory infiltrates, lymphoid hyperplasia, and airway-centered fibrosis, etc.) in the cases otherwise acceptable for UIP. Pathologists are often in a difficult position to determine whether the presence of these minor changes would disqualify the diagnosis of UIP or not. There have been widely differing opinions and approaches in this regard even among expert pulmonary pathologists.

In this backdrop of diagnostic conundrum, a bold concept was proposed to consider UIP as a stand-alone diagnostic entity, encompassing not only IPF but also other secondary processes, based on the presence of radiologic or histopathologic features of UIP [17]. It was argued that UIP pattern of fibrosis in other ILDs (especially fibrotic HP and CTD-associated ILD) as well as in IPF is associated with unfavorable clinical outcomes [18]. In addition, acute exacerbation may occur in rheumatoid arthritis–associated ILD and fibrotic HP with similarly dismal outcome to that seen in IPF [19,20]. The significant similarities between IPF and other secondary conditions with UIP features in clinical behavior, pathogenic pathways, and the efficacy of anti-fibrotic therapy were cited as the justification for a lumping approach in this proposal. A radiologic study reported that the presence of honeycomb change alone predicted an IPF-like mortality in patients with other conditions (fibrotic HP, CTD-ILD, and unclassifiable ILD), which could support this notion [21].
Concept of PPF: green light for lumping fibrosing ILD diagnoses

There are many types of idiopathic and secondary fibrosing ILDs, in which IPF is considered as the prototype [22]. Non-IPF fibrosing ILDs include other IIPs (e.g., fibrotic NSIP, DIP, and AIP, etc.), autoimmune ILDs (e.g. rheumatoid arthritis-associated ILD), exposure-related ILDs (e.g. HP, occupational exposures, medications), pulmonary Langerhans cell histiocytosis and sarcoidosis, among others [7]. Recently, fibrosing ILDs have been studied as a group, given their clinical, radiological, and histopathological overlap. In this approach, a subset of non-IPF fibrosing ILDs was found to show a very similar clinical phenotype to that of IPF, characterized by progressive worsening of respiratory symptoms, declining lung function, developing acute exacerbation, and resistance to conventional therapy, ultimately resulting in high mortality [23-26]. In 2017, a term “progressive fibrosing ILDs” was first coined to this group of cases but an alternative term “progressive pulmonary fibrosis” (PPF) was endorsed in the 2022 ATS/ERS/JRS/ALAT clinical practice guideline [7,26]. In this 2022 statement, PPF was officially defined as an ILD of known or unknown etiology, other than IPF, with radiologic evidence of pulmonary fibrosis as well as meeting the set of specific clinical/radiological criteria including worsening respiratory symptoms and a certain degree of progression in pulmonary function abnormalities and/or radiological evidence of disease progression, occurring within the past year [7]. In this guideline, PPF is clearly stated as an entity of prognostic significance but not a specific diagnosis per se. The conceptual evolution to PPF over time is illustrated in Fig. 3.

Clinical trials have evaluated the efficacy of antifibrotics approved for IPF in the non-IPF patients who underwent progressive fibrosis, regardless of the type of underlying ILDs. Nintedanib therapy in the INBUILD trial resulted in a significant reduction in the annual decline of FVC in patients with PPF [27]. Pirfenidone therapy in two randomized control trials also showed a reduced FVC decline in some PPF patients [28,29]. Based on the results of these trials, the 2022 ATS clinical practice guidelines gave a conditional recommendation for nintedanib for the treatment of PPF in patients who have failed standard management, but not for pirfenidone given the lack of sufficient evidence [7].

This novel approach to treat ILD based on the disease behavior (i.e., PPF) without consideration of the specific underlying diagnosis will have a broader, potentially deleterious, influence in many aspects of ILD. The ATS guideline on PPF explicitly stated that this new concept of PPF should not discourage clinicians from their rigorous effort to identify the underlying type of ILD before antifibrotic therapy. In reality, however, it is most likely that the incentive of establishing a definitive diagnosis of

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**Fig. 3.** The idiopathic interstitial pneumonia (IIP) classification established in 2000s is evolved from the less well-defined idiopathic pulmonary fibrosis (IPF) that encompassed various histologic patterns. Progressive pulmonary fibrosis is the current official practice guideline by American Thoracic Society (ATS)/European Respiratory Society (ERS)/Japanese Respiratory Society (JRS)/Latin American Thoracic Association (ALAT) can be the manifestation by many fibrotic lung diseases other than IPF. AIP, acute interstitial pneumonia; CFA, cryptogenic fibrosing alveolitis; COP, cicatrical organizing pneumonia; COP, cryptogenic organizing pneumonia; CTD-ILD, connective tissue disease-associated interstitial lung disease; DIP, desquamative interstitial pneumonia; F-HP, fibrotic hypersensitivity pneumonitis; F-NSIP, fibrotic nonspecific interstitial pneumonia; FOP, fibrotic organizing pneumonia; LCH, Langerhans cell histiocytosis; PPFE, pleuroparenchymal fibroelastosis; RBILD, respiratory bronchiolitis interstitial lung disease; UIP, usual interstitial pneumonia.
the underlying ILD would be markedly reduced if not completely vanished. A few studies attempted to analyze the clinical trials data based on specific ILD subgroups within PPF but did not have enough power to provide evidence of benefit in different subgroups [30,31]. The criteria of PPF do not include pathologic findings, but it may be helpful to evaluate any histopathologic findings that correlate with progressive fibrosis. While UIP histopathologic pattern is an obvious leading candidate given its well-known compelling evidence, characterization of other histologic features that could predict the PPF phenotype has yet to be established [17].

**Familial pulmonary fibrosis: a clue to decode molecular pathogenesis of fibrosing ILD**

Familial cases of ILDs were reported in 1907, which suggested a possibility of genetic factors in ILDs [32]. The concept of familial IPF was first introduced in the 2000 ATS statement with the following definition: at least two members of a primary biological family (parent, child, sibling) with clinical features of IPF and histologic confirmation [3]. Though not explicitly recommended in later ATS statements, the histology requirement in the criteria was eventually dropped in practice, which is in keeping with the trends of bypassing the biopsies and making IPF diagnosis based on HRCT at least in more classic cases of IPF on clinical ground. Familial clustering of non-IPF fibrosing ILDs have also been observed. Other names have been used for this group of diseases, including familial/inherited interstitial lung disease, familial interstitial pneumonia, familial pulmonary fibrosis, and familial IPF, if limited to idiopathic cases. The plethora of terminology has clouded the literature and hampered clearer communication. In this review, the term familial pulmonary fibrosis (FPF) will be used that encompasses all types of pulmonary fibrosis including IPF.

The frequency of FPF may be as high as 20% of patients with pulmonary fibrosis according to the previous studies reported in the literature [33-38]. Among the subtypes of fibrotic ILD, IPF most frequently had a family history of pulmonary fibrosis (20%–25%), followed by chronic HP (14%–17%), and CTD-related ILD (3%–8%) [38-41]. Of note, relatives in the same family may show different subtypes of fibrotic ILD [42,43]. Radiographic screening studies revealed some radiological abnormalities in 15 to 31% of the asymptomatic relatives of patients with known pulmonary fibrosis, suggesting even higher prevalence of FPF than suspected [44-46].

The remarkable advances in molecular technology led to several large-scale, population-based studies on pulmonary fibrosis to identify genetic risk variants that could be associated with crucial pathogenetic mechanisms in pulmonary fibrosis. Two broad categories of the variants associated with pulmonary fibrosis have been recognized based on their frequency in the population: common and rare genetic variants. Common genetic variants typically represent single nucleotide polymorphisms (SNPs) and confer a smaller effect than rare variants as the impact on disease risk tends to be inversely proportional to the frequency of a variant within the population [47]. It has been well accepted that SNPs may contribute to overall risk but not entirely sufficient to cause disease on their own. On the other hand, rare variants often demonstrate cosegregation in FPF kindreds, suggesting a causal relationship.

Linkage analysis and genome-wide association studies have identified numerous common genetic variants associated with IPF. Gain-of-function promoter variant rs35705950 in the promoter region of the MUC5B gene is most widely recognized in association with pulmonary fibrosis. A study reported that this genetic variant is identified in 34% of the subjects with familial IPF, 38% of those with sporadic IPF, and 9% of the control subjects [48]. Rare genetic variants in pulmonary fibrosis were mainly implicated in dysfunctional surfactant metabolism and telomere maintenance. Genes involved in abnormal surfactant metabolism include SFTPC, SFTPA1/2, and ABCA3 [40]. The pattern of inheritance is autosomal dominant for SFTPC and SFTPA1/2 and autosomal recessive for ABCA3 [49]. Disease onset has a wide range from infancy to late adulthood in families with a rare surfactant-related gene variant; histologic and radiologic features of ILD are also diverse with the features of UIP, NSIP, and DIP [50-52]. No extrapulmonary manifestations are present in these patients as surfactant production is limited to the lung. The rare genetic variants associated with deranged telomere maintenance account for approximately 25% of FPF kindreds and involve TERT, TERC, and numerous other genes [53,54]. Unlike the gene variants associated with surfactant dysfunction, these variants of telomere-related genes often cause extrapulmonary systemic manifestations (known as short telomere syndrome or other related terms) [55]. Dyskeratosis congenita (DC) is one of the best known telomeropathies due to homozygous telomere-related gene mutations resulting in extreme telomere shortening. DC causes pulmonary fibrosis (20%) as well as life-threatening bone marrow failure (80%) at pediatric age or in very early adulthood. DC often shows various cutaneous or mucosal manifestations including nail dystrophy, abnormal skin pigmentation, and oral leukoplakia [55,56].

The patients with heterozygous telomere–related gene muta-
tions also often develop pulmonary fibrosis including IPF (50%), fibrotic HP (7%–12%) connective tissue disease–associated ILD (2%–3%), or other IIDs (14%–18%) with similar extrapulmonary manifestations of DC but at an older age than in DC [43,57,58]. Interestingly, about 10% of adult-onset sporadic IPF, chronic HP, and rheumatoid arthritis-related ILD patients have rare telomere-related gene variants [59-62]. Short telomere length is often seen in FPF cases and a frequent finding in sporadic cases of pulmonary fibrosis as compared to control subjects, suggesting a possibility that short telomere length might be a potential cause of pulmonary fibrosis [63].

Despite a remarkable progress in identifying key genetic features associated with FPF (and some sporadic fibrosing ILD cases), characterization of the matching pathologic features in FPF seemed to have lagged behind. Steele et al published a study in 2005 with histopathologic assessment in some of their cases from families with IIDs and found significant heterogeneity within a given family, showing more than one histopathologic subtype of IIP in 45% of family pedigrees [42]. A comprehensive histopathologic study by Leslie et al compiled the findings to differentiate familial and sporadic IIP cases in an effort to characterize the features of familial IIP, primarily focusing on familial IPF [64]. Although most of their patients had some histopathologic features associated with UIP, up to 60% of them did not qualify as UIP, mainly due to lack of the temporal heterogeneity of fibrosis, one of the most important histologic criteria for UIP [64]. Most of these cases ended up in the unclassifiable fibrotic ILD category [64]. Prevailing notion in the past has been that familial and sporadic IPF cases do not have distinguishable clinical or histological features other than earlier onset in familial IPF [6]. However, this 2012 study by Leslie et al. [64] suggested that there may be some histopathologic features differentiating sporadic and familial fibrotic lung diseases. Based on this observation, they concluded that unclassifiable pattern of lung fibrosis should raise a possibility of FPP or familial IPF [64]. They also suggested that less than classic histopathologic features of UIP could still be compatible with familial IPF in an appropriate clinical context [64].

**AI in diagnosis of interstitial lung diseases**

The application of AI tools on radiologic images for characterization of indeterminate lung nodules, fibrotic lung diseases, and lung cancer risk stratification has been well studied and documented in the literature. As compared to radiology arena, digital pathology (DP) by WSI started more recently and most AI approach has been applied to cancers and biomarker arena of other organs (such as breast and prostate). In the lungs, AI tools have also been more widely applied to cancers than to ILDs, partly due to the current tendency of relying on high-resolution CT and bypassing surgical lung biopsies for diagnosis of ILDs, especially after the 2013 ATS/ERS guidelines.

As there are many clinically as well as morphologically ambiguous cases of ILD, AI-assisted diagnosis may offer a crucial contribution by detecting some features that are not discernable by traditional diagnostic methods. Moreover, AI approach may resolve some interobserver variability in diagnosing ILDs that has been well-recognized even among the expert pulmonary pathologists. It is inherently subjective to interpret the basic histopathologic parameters for ILD diagnosis (such as architectural distortion or scarring, honeycomb change, traction bronchiectasis, distribution of fibrosis, degree of inflammation, fibroblast foci, presence of granulomas, to name a few). Thus, more objective interpretation and quantitation of these parameters would be ideal by appropriate training and development of AI algorithm. Once AI-assisted diagnostic tool becomes widely available, it may mitigate the challenges encountered by many hospitals without direct access to experienced pathologists who are well versed in ILD diagnoses. Finally, some advanced AI technology (e.g. DL-based AI via unsupervised feature learning) might offer new insights into ILDs by elucidating previously unknown histopathologic features associated with progressive clinical behavior as in PPF cases. Such an approach, however, requires a large number of surgical lung biopsy cases with clinical annotation, which became scarce these days due to the changing practices as elaborated in the earlier sections. Multi-institutional collaboration might be needed to form a consortium for securing sufficient database to develop appropriate AI model.

A complete coverage of AI tools in DP is beyond the scope of the present review and only a brief introduction of basic concepts in the AI approach will be provided, followed by summary of several original studies in the literature on predicting pathologic diagnosis and prognosis of ILDs, or evaluation of histopathologic biomarkers in IPF with AI technology (Table 1).

AI is a broad discipline with multiple approaches to construct a model for a particular task. There are two common ways to extract feature representation for building an AI model: (1) DL-based unsupervised feature learning, (2) hand-crafted approach [65]. Convolutional neural network (CNN) is a type of DL approach suited for low-level tasks such as detecting, identifying, and classification of images. DL-based unsupervised feature learning is favored in low-level tasks such as cell and lung tumor detection and classification. This is useful since visual confirmation of the result is sufficient and does not require interpretation.
of selected features. In the hand-crafted approach, relevant features from data are manually selected. The domain knowledge is employed for features engineering, by close collaboration between pathologists and machine learning engineers to construct appropriate AI models. In contrast to DL-based unsupervised feature learning, hand-crafted based models offer some interpretability through incorporating the expertise of subject matter experts. Many high-level tasks such as prognosis or treatment response prediction which require a certain level of interpretability and hence hand-crafted, domain-inspired features may be favored by medical community to construct these models. Integration of DP in clinical workflow across major institutions poses a huge challenge, due to organizational structures, the cost of initial set-up, requirements of advanced security systems in hospitals and demands for storage of big data. Currently, most existing AI tools have been validated on retrospective data, which does not represent the current, real-world scenarios. Validating the algorithms in randomized controlled trials and prospective studies will be a crucial step towards clinical adoption of these AI tools.

Makela et al. [66] reported a study using AI to count fibroblast foci, a known prognostic factor in IPF, and other parameters to evaluate their prognostic significance. In this study, they trained a CNN to quantify fibroblast foci, interstitial mononuclear inflammation, and intra-alveolar macrophages to analyze the association of these parameters with survival [66]. Interstitial mononuclear inflammation and intra-alveolar macrophages were associated with poor prognosis, while increased fibroblast foci were associated with longer survival.

Uegami et al. [67] proposed an original method to develop DL models for extracting pathologically significant findings based on expert pathologist’s perspective with a small annotation effort. Their method named MIXTURE (huMan-In-the-loop eXplainable artificial intelligence Through the Use of REcurrent training) consisted three steps including (1) creating feature extractors for tiles from whole slide images using self-supervised learning, (2) clustering similar looking tiles based on output features, followed by integration of the pathologically synonymous clusters by pathologists, and (3) creation of DL models to classify tiles into pathological findings by using the integrated clusters as labeled data. They developed three models for different magnification. Their model predicted the diagnosis of UIP with a high accuracy, which was not possible to achieve without the step of integration of findings by pathologists. They proposed this model as the prototype for explainable AI that can collaborate with humans.

Testa et al. [68] reported a study that performed an automated digital quantification of pulmonary fibrosis in human histopathology specimens. They conducted a pilot study to analyze a small number of specimens from patients with Hermansky-Pudlak syndrome pulmonary fibrosis (n = 3) or IPF (n = 9) using digital images of serial lung sections stained with picrosirius red, alcian blue or anti-CD68 antibody. Dedicated software was used to automatically quantify fibrosis, collagen, and macrophage content. Automated fibrosis quantification based on parenchymal tissue density and fibrosis score measurements were compared to the pulmonary function test values or Ashcroft score, a numerical scale with grades from 0 to 8, of the amount of fibrotic tissue in histological samples devised by Ashcroft et al. [69]. A high correlation coefficient was found between some automated quantification measurements and lung function values in the sample groups. They concluded that computerized image analysis can offer accurate, reader-independent pulmonary fibrosis quantification in human histopathology samples for various parameters such as fibrosis, collagen content, and immunostained cells. This approach may enhance the available tools to quantify and study fibrotic ILDs.

Table 1. Published original research on interstitial lung diseases with AI-based methods

<table>
<thead>
<tr>
<th>Study</th>
<th>Purpose of study</th>
<th>Method</th>
<th>Country</th>
<th>No. of cases</th>
<th>Main finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Makela et al. (2021)</td>
<td>Quantitation of histologic parameters for survival analysis in IPF</td>
<td>Deep CNN</td>
<td>Finland</td>
<td>71 IPF</td>
<td>Increased FF is associated with poor prognosis—interstitial mononuclear infiltrate &amp; intra-alveolar macrophage with longer survival.</td>
</tr>
<tr>
<td>Uegami et al. (2022)</td>
<td>Prediction of UIP diagnosis</td>
<td>MIXTURE</td>
<td>Japan</td>
<td>231 (UIP+non-UIP) cases; 715 WSI</td>
<td>A model approach to differentiate diseases by AI with collaboration with humans based on expert pathologists’ input.</td>
</tr>
<tr>
<td>Testa et al. (2021)</td>
<td>Pilot study to quantify fibrosis in HPSPF &amp; IPF</td>
<td>Automated quantitation of fibrosis with dedicated software</td>
<td>USA</td>
<td>3 HPSPF &amp; 9 IPF</td>
<td>Their automated image analysis offered accurate, reader-independent pulmonary fibrosis quantitation in HPSPF and IPF groups.</td>
</tr>
</tbody>
</table>

AI, artificial intelligence; CNN, convoluted neural network; IPF, idiopathic pulmonary fibrosis; FF, fibroblastic foci; MIXTURE, huMan-In-the-loop eXplainable artificial intelligence Through the Use of REcurrent training; UIP, usual interstitial pneumonia; WSI, whole slide imaging; HPSPF, Hermansky-Pudlak syndrome pulmonary fibrosis.
CONCLUSION

Since Dr. Liebow introduced the diagnosis of UIP/IPF as a type of ILDs more than 50 years ago, histopathologic criteria have been refined and established. Antifibrotic therapy recently became available for UIP/IPF patients and brought modest improvement in its otherwise dismal clinical course. Partly based on the efficacy of antifibrotic therapy in other types of fibrosing ILDs, albeit limited, a proposal of UIP as a stand-alone entity was made to encompass all fibrosing ILDs with UIP pattern under the broad umbrella of UIP to be treated as UIP/IPF patients. Likewise, a concept of PPF that includes many types of fibrosing ILDs was endorsed as a prognostic entity (but not a specific diagnosis) by an international committee that also cited the similar therapeutic approach to that in UIP/IPF as the main reason for creating this category. Common and rare genetic variants identified in fibrosing ILD patients shed light in molecular pathways implicated in sporadic as well as familial cases of fibrosing ILDs. Whole slide imaging and AI might open the door to a new era provided accumulation of big histopathologic database and collective effort in this approach support the progress.

Ethics Statement
Not applicable.

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: ESY, JHR. Data curation: ESY, PW. Supervision: ESY. Writing—original draft: ESY, PW. Writing—review & editing: ESY, PW, JHR. 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
17. Selman M, Pardo A, Wells AU. Usual interstitial pneumonia as a


Diagnosis of ILD

Tumor-infiltrating T lymphocytes evaluated using digital image analysis predict the prognosis of patients with diffuse large B-cell lymphoma

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Background: The implication of the presence of tumor-infiltrating T lymphocytes (TIL-T) in diffuse large B-cell lymphoma (DLBCL) is yet to be elucidated. We aimed to investigate the effect of TIL-T levels on the prognosis of patients with DLBCL. Methods: Ninety-six patients with DLBCL were enrolled in the study. The TIL-T ratio was measured using QuPath, a digital pathology software package. The TIL-T ratio was investigated in three foci (highest, intermediate, and lowest) for each case, resulting in TIL-T–Max, TIL-T–Intermediate, and TIL-T–Min. The relationship between the TIL-T ratios and prognosis was investigated. Results: When 19% was used as the cutoff value for TIL-T–Max, 72 (75.0%) and 24 (25.0%) patients had high and low TIL-T–Max, respectively. A high TIL-T–Max was significantly associated with lower serum lactate dehydrogenase levels (p < .001), with patient group who achieved complete remission after R-CHOP therapy (p < .001), and a low-risk revised International Prognostic Index score (p < .001). Univariate analysis showed that patients with a low TIL-T–Max had a significantly worse prognosis in overall survival compared to those with a high TIL-T–Max (p < .001); this difference remained significant in a multivariate analysis with Cox proportional hazards (hazard ratio, 7.55; 95% confidence interval, 2.54 to 22.42; p < .001). Conclusions: Patients with DLBCL with a high TIL-T–Max showed significantly better prognosis than those with a low TIL-T–Max, and the TIL-T–Max was an independent indicator of overall survival. These results suggest that evaluating TIL-T ratios using a digital pathology system is useful in predicting the prognosis of patients with DLBCL.

Key Words: Diffuse large B-cell lymphoma; Tumor infiltrating lymphocytes; T lymphocytes; Digital pathology

Diffuse large B-cell lymphoma (DLBCL) is the most common malignant lymphoma in adults [1,2]. Currently, the rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) regimen is used as the first-line chemotherapy for patients with DLBCL. Approximately 70% of the patients achieve long-term remission; however, the remaining patients die as a result of relapse or refractory disease [3-5]. Although immunotherapy is effective for several other solid tumors, only a subset of patients with DLBCL responds to immunotherapy [6,7]. T lymphocytes, macrophages, natural killer cells, stromal cells, blood vessels, and the extracellular matrix act complexly in the mechanism of immunotherapy. Thus, it is important to increase our understanding of the tumor microenvironment (TME) to establish an effective immunotherapeutic strategy [8-12]. In particular, chimeric antigen receptor T-cell (CAR-T) therapy has recently been used to treat patients with DLBCL [13,14], which significantly increases the importance of T lymphocytes in the TME of DLBCL.

Previous studies have speculated that T lymphocytes may be associated with the clinical course of DLBCL. One study demonstrated that a higher percentage of tumor-infiltrating T lymphocytes (TIL-T) was correlated with better patient survival [15]. However, results vary with respect to the effect of CD3-positive T-cell infiltration on patient outcomes [16], and more researchers have reported the prognostic value of a specific subset of TIL-T, namely CD4-positive T cells, in DLBCL [17,18].
The widespread use of digital pathology systems has made it possible to objectively and accurately evaluate histological analyses performed subjectively by pathologists. In this study, we used immunohistochemistry (IHC) and a digital pathology system to precisely measure TIL-T levels in DLBCL, not otherwise specified (NOS), and investigated its association with patient prognosis. We also revealed that a high TIL-T ratio is an independent favorable prognostic factor in patients with DLBCL NOS.

**MATERIALS AND METHODS**

**Patient selection**

Ninety-six consecutive patients diagnosed with DLBCL NOS at Samsung Medical Center, Seoul, Korea, from January to December 2018, were enrolled in this study. Small biopsy and diagnostic resection were performed in 66 and 30 patients, respectively. The patients' clinical and pathological information was evaluated by reviewing their electronic medical records. There were 48 males and 48 females, with a male-to-female ratio of 1:1. The median age of the patients was 61 years (range, 5 to 86 years).

According to the Ann Arbor stage, 52 patients (54.2%) were stage I–II and 44 (45.8%) were stage III–IV. The number of cases subtyped by cells of origin (COO) [19] was 32 (33.3%) for the germinal center B-cell (GCB) type and 64 (66.7%) for the non-GCB type. At the time of diagnosis, serum lactate dehydrogenase (LDH) levels were elevated above the normal range in 39 patients (40.6%). All patients initially received R-CHOP chemotherapy, except for five patients who either died or were lost to follow-up before they could be treated. Fifty of the 91 treated patients achieved complete remission (CR) after the standard six course of R-CHOP. Among the 41 patients who did not, nine were only treated with R-CHOP, 17 went on to receive second and/or third-line chemotherapy, and 15 had autologous stem cell transplantation. Based on the revised International Prognostic Index (R-IPI)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients (%)</th>
<th>TIL-T–Max</th>
<th>TIL-T–Intermediate</th>
<th>TIL-T–Min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High (n=72, 75.0%)</td>
<td>Low (n=24, 25.0%)</td>
<td>p-value</td>
</tr>
<tr>
<td>Age (yr) ≤ 60</td>
<td>48 (50.0)</td>
<td>39 (61.0)</td>
<td>9 (45.5)</td>
<td>&gt; .999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32 (50.8)</td>
<td>16 (53.3)</td>
<td>&gt; .999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 (33.3)</td>
<td>30 (50.0)</td>
<td>&gt; .999</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>38 (39.6)</td>
<td>30 (76.2)</td>
<td>&gt; .999</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>58 (60.4)</td>
<td>42 (72.4)</td>
<td>&gt; .999</td>
</tr>
<tr>
<td>Ann Arbor stage</td>
<td>Low (I–II)</td>
<td>52 (62.5)</td>
<td>43 (82.7)</td>
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<tr>
<td></td>
<td>High (III–IV)</td>
<td>44 (45.8)</td>
<td>29 (56.9)</td>
<td>&gt; .999</td>
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<tr>
<td>COO</td>
<td>GCB</td>
<td>32 (33.3)</td>
<td>25 (78.1)</td>
<td>&gt; .999</td>
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<tr>
<td></td>
<td>Non-GCB</td>
<td>64 (66.7)</td>
<td>47 (71.9)</td>
<td>&gt; .999</td>
</tr>
<tr>
<td>Serum LDH level</td>
<td>Low</td>
<td>57 (59.4)</td>
<td>52 (93.0)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>39 (40.6)</td>
<td>20 (57.1)</td>
<td>&lt; .001</td>
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<tr>
<td>Treatment</td>
<td>CR after R-CHOP</td>
<td>50 (51.2)</td>
<td>45 (90.0)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>Non-CR</td>
<td>46 (47.9)</td>
<td>27 (53.6)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>R-CHOP only</td>
<td>9</td>
<td>41 (100.0)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>+ 2nd and/or 3rd line CTx</td>
<td>17</td>
<td>41 (100.0)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>+ AutoSCT</td>
<td>15</td>
<td>19 (100.0)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>Pre-treatment</td>
<td>5</td>
<td>19 (100.0)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>R-IPI</td>
<td>Low risk (0–2)</td>
<td>63 (65.6)</td>
<td>55 (84.6)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>High risk (3–5)</td>
<td>33 (34.4)</td>
<td>17 (35.4)</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

TIL-T, tumor-infiltrating T lymphocyte; COO, cells of origin; GCB, germinal center B-cell-like; LDH, lactate dehydrogenase; CR, complete remission; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; CTx, chemotherapy; AutoSCT, autologous stem cell transplantation; R-IPI, revised International Prognostic Index.
Cho Y et al. [20], which takes into consideration the number of negative prognostic factors present at the time of diagnosis (age > 60 years, stage III/IV disease, elevated LDH level, Eastern Cooperative Oncology Group performance status ≥ 2, more than one extranodal site of disease), cases were stratified into low-risk group (0–2 negative factors) and high-risk group (3–5 negative factors). Sixty-three (65.6%) cases were low risk and 33 (34.4%) were high risk. The clinicopathological characteristics of the patients are summarized in Table 1. The median follow-up period was 1,181 days (range, 2 to 1,894 days).

**Computational image analysis**

IHC for CD3 (Polyconal, Dako, Glostrup, Denmark) and CD20 (clone L26, Dako), performed at the time of diagnosis, was used for TIL-T ratio evaluation. All slides were scanned using a Pannoramic 1000 scanner (3DHistech, Budapest, Hungary). The INFINITT DPS (INFINITT Healthcare, Seoul, Korea) was used as the image-viewing system. Two independent pathologists (Y.C. and J.C.), without prior knowledge of the patients’ clinical outcomes, thoroughly evaluated tumor areas with dense B-cell populations, as detected by CD20 IHC. Among the differing densities of CD3-positive lymphocytes, the areas with the highest, lowest, and intermediate densities of CD3-positive cells in each case were manually selected. Areas with the highest and the lowest densities were first identified, then those areas were excluded from determining the intermediate density area. Intermediate density was taken from an area occupying the largest proportion of the tumor with similar level of CD3-positive lymphocyte density. Disagreements were resolved by consensus, and the consensus foci were adopted as final. All the selected areas were then photographed in a 400 × 400 μm square image. The image files were processed using QuPath software [21]. Cell-detection was conducted using QuPath’s built-in “Positive cell detection” to determine the ratio of TIL-T to total cell of each focus (Fig. 1). The TIL-T ratios of the areas with the highest, lowest, and intermediate CD3-positive cell densities were denoted as TIL-T–Max, TIL-T–Min, and TIL-T–Intermediate, respectively.

**Statistical analysis**

Comparisons between clinical features and TIL-T ratios were
performed using Pearson’s chi-square test. Overall survival (OS) was defined as the time from the date of diagnosis to death from any cause. Survival distribution was compared using the Kaplan-Meier method and log-rank test. Prognostic variables associated with OS were examined by univariate analysis using the Cox proportional hazards regression model. Only variables significantly associated with survival were included in the multivariate regression analyses. Statistical analyses were performed, and graphics were obtained using R ver. 4.1.3 (https://www.r-project.org). A p-value < .05 was considered statistically significant.

RESULTS

Evaluation of TIL-T ratio and comparison with clinicopathological parameters

TIL-T–Max, TIL-T–Min, and TIL-T–Intermediate were calculated as described above. The receiver operating characteristic curve analysis was used to determine the optimal cutoff value with respect to the OS of patients for each TIL-T ratio (Fig. 2). Cutoff points between high and low TIL-T ratios were 19%, 11%, and 13% for TIL-T–Max, TIL-T–Intermediate, and TIL-T–Min, respectively. With TIL-T–Max as the criterion, 72 (75.0%) had > 19% TIL-T–Max and 24 (25.0%) had < 19% TIL-T–Max; with TIL-T–Intermediate, 64 (66.7%) patients had a high TIL-T–Intermediate and 32 (33.3%) had a low TIL-T–Intermediate; and with TIL-T–Min, 36 (37.5%) had a high TIL-T–Min and 60 (62.5%) had a low TIL-T–Min (Table 1). The mean values for the TIL-T–Max, –Intermediate, and –Min were 41.6% (range, 0.3% to 98.5%; standard deviation [SD], ± 27.72), 26.3% (range, 0.2% to 97.5%; SD, ± 23.18), and 14.4% (range, 0.1% to 88.8%; SD, ±16.75), respectively. The clinicopathological parameters associated with TIL-T ratios are listed in Table 1. A high TIL-

Fig. 2. Receiver operating characteristic curves of tumor-infiltrating T lymphocyte (TIL-T) ratios. The cutoff values of 19%, 11%, and 13% for TIL-T–Max (A), TIL-T–Intermediate (B), and TIL-T–Min (C), respectively, were inferred from these analyses.
T–Max was significantly associated with serum LDH levels within the normal range ($\chi^2 = 17.634$, degrees of freedom [df] = 1, $p < .001$), with the group of patients that achieved CR after initial R-CHOP therapy ($\chi^2 = 10.908$, df = 1, $p < .001$) and with low-risk R-IPI ($\chi^2 = 12.945$, df = 1, $p < .001$). A high TIL-T–Intermediate was also significantly associated with low serum LDH levels ($\chi^2 = 8.211$, df = 1, $p = .004$), CR after R-CHOP ($\chi^2 = 9.647$, df = 1, $p = .002$), and a low risk of R-IPI ($\chi^2 = 4.208$, df = 1, $p = .040$). The TIL-T–Min was not significantly associated with any of the clinical variables.

**Survival analysis**

Patients with a high TIL-T–Max showed a significantly longer OS ($p < .001$) (Fig. 3A). This trend was also observed in the high TIL-T–Intermediate ($p < .001$) (Fig. 3B) and high TIL-T–Min ($p < 0.001$) (Fig. 3C). According to the univariate Cox proportional hazards analysis, a shorter OS was significantly associated with a high Ann Arbor stage, and a longer OS was significantly associated with a serum LDH level within the normal range, patients achieving CR after R-CHOP therapy, and low-risk R-IPI (Table 2). Univariate analysis revealed that patients with low TIL-T–Max had a significantly worse prognosis in OS compared to those with high TIL-T–Max (hazard ratio [HR], 15.8; 95% confidence interval [CI], 6.20 to 40.25; $p < .001$) (Table 2), and those with low TIL-T–Intermediate compared to high TIL-T–Intermediate (HR, 6.91; 95% CI, 2.85 to 16.74; $p < .001$) (Table 2). This trend was also observed in the TIL-T–Min; patients with a low TIL-T–Min had worse OS than those with a high TIL-T–Min (HR, 7.98; CI, 1.87 to 33.95; $p = .005$) (Table 2). In multivariate analysis, a low TIL-T–Max was found to be an independent unfavorable prognostic factor in patients with DLBCL NOS (HR, 7.55; 95% CI, 2.54 to 22.42; $p < .001$). The same trend was also observed in TIL-T–Intermediate (HR, 2.96; 95% CI, 1.17 to 7.53; $p = .022$) (Table 2, Fig. 4).

**DISCUSSION**

In this study, we used digital image analysis to evaluate the
TIL-T predicts the prognosis of DLBCL

Exact ratio of TIL-T in patients with DLBCL NOS. Low TIL-T ratios in the TIL-T–Max, TIL-T–Intermediate, and TIL-T–Min were associated with shorter OS and were found to be independent prognostic factors in patients with DLBCL.

Previous studies have focused on the prognostic significance of the total TIL-T ratio in patients with DLBCL. An immunohistochemical study of TIL-T in DLBCL showed no correlation between survival and TIL-T percentage [16]. Another study found that the percentage of CD3+ cells (total T cells) had no impact on DLBCL patient outcomes [18]. However, Xu et al. (2001) [15] found that a TIL-T ratio > 20% showed a favorable prognosis compared to a TIL-T ratio < 20%. Another study reported that patients with high CD3+ TIL-T levels (> 45%) had better event-free survival and OS rates, although CD4+ TIL-T was statistically more relevant than CD3+ TIL-T in predicting patient outcomes [17]. All the studies mentioned above were conducted using flow cytometry analyses of fresh biopsy tissue, except for one by Lippman et al. (1990) [16]. Flow cytometric analysis of the TIL-T ratio in DLBCL specimens may have contributed to these contradictory results. Flow cytometric analysis, a rapid multi-parametric analysis of single cells in solution, lacks morphological correlation. Sampling errors such as the inclusion of normal lymphoid tissue adjacent to the malignant tumor area can influence the sensitivity of flow cytometry and result in reports that are not representative of the actual TME. Also, flow cytometric analysis cannot take into consideration of the differing areas of densities of TIL-Ts in one tumor specimen. In our study, formalin-fixed paraffin-embedded tissue specimens were used for IHC staining, and morphological evaluation of the whole slide was performed to detect the actual focus of T-lymphocyte infiltration among diffuse neoplastic B-cell populations. Moreover, the correlation between the TIL-T ratio and patient outcome was more statistically significant in the TIL-T–Max, suggesting that the clinical behavior of DLBCL may be more

Table 2. Univariate and multivariate analyses of factors associated with overall survival in 96 diffuse large B-cell lymphoma, not otherwise specified patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison/referent</th>
<th>Univariate</th>
<th>Multivariate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>TIL-T–Max</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>&gt; 60/≤ 60</td>
<td>0.62</td>
<td>0.28–1.40</td>
</tr>
<tr>
<td>Sex</td>
<td>Male/Female</td>
<td>1.42</td>
<td>0.61–3.33</td>
</tr>
<tr>
<td>Ann Arbor stage</td>
<td>III–IV/II</td>
<td>4.14</td>
<td>1.64–10.43</td>
</tr>
<tr>
<td>Cell-of-origin</td>
<td>Non-GCB/GCB</td>
<td>1.60</td>
<td>0.64–4.03</td>
</tr>
<tr>
<td>Serum LDH level</td>
<td>Low/High</td>
<td>0.18</td>
<td>0.07–0.45</td>
</tr>
<tr>
<td>Treatment</td>
<td>Non-CR/CR after R-CHOP</td>
<td>37.42</td>
<td>5.04–277.70</td>
</tr>
<tr>
<td>R-IPI</td>
<td>Low risk/High risk</td>
<td>0.15</td>
<td>0.06–0.37</td>
</tr>
<tr>
<td>TIL-T–Max</td>
<td>Low/High</td>
<td>15.81</td>
<td>6.20–40.25</td>
</tr>
<tr>
<td>TIL-T–Intermediate</td>
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<tr>
<td>Age (yr)</td>
<td>&gt; 60/≤ 60</td>
<td>0.62</td>
<td>0.28–1.40</td>
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<td>TIL-T–Intermediate</td>
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<td>6.91</td>
<td>2.85–16.74</td>
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<tr>
<td>TIL-T–Min</td>
<td>Low/High</td>
<td>7.98</td>
<td>1.87–33.96</td>
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</table>

HR, hazard ratio; CI, confidence interval; TIL-T, tumor-infiltrating T lymphocyte; GCB, germinal center B-cell-like; LDH, lactate dehydrogenase; CR, complete remission; R-IPI, revised International Prognostic Index.
**Fig. 4.** Associations between survival probability and tumor-infiltrating T lymphocyte (TIL-T) ratios. Hazards ratios are shown separately for TIL-T–Max (A), TIL-T–Intermediate (B), and TIL-T–Min (C). Hazard ratio and p-values were corrected by Ann Arbor stage, serum lactate dehydrogenase (LDH) levels, patient treatment, and the revised International Prognostic Index (R-IPI) score. AIC, Akaike information criterion; CR, complete remission; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. (Continued to next page)
TIL-T predicts the prognosis of DLBCL
•

accurately reflected by the TIL-T ratio at the densest T lymphocyte infiltration. This finding underscores the importance of histological assessment in determining the TIL-T ratio in patients with DLBCL. TIL-T levels of tumor are mainly determined by the intrinsic immunogenicity of tumor cells. In partial areas of tumors, it is possible that TIL-T levels are lowered by other factors, such as immune checkpoints. Therefore, among various TIL-T levels in tumors, it could be considered that TIL-T–Max is most likely to accurately reflect the intrinsic immunogenic capacity of tumor cells, which may have contributed to a more precise predictions of a patient prognosis. Further study is required to confirm the prognostic value of the TIL-T–Max and to substantiate the hypothesis behind it.

In our results, the high TIL-T ratio group according to the TIL-T–Max showed no correlation with patient age, sex, or COO of tumor. The Ann Arbor stage also showed no statistically significant association with the TIL-T–Max. However, serum LDH levels were significantly elevated in patients with a low TIL-T–Max. This suggests that the degree of TIL-T is determined by the immunogenicity of the tumor cells rather than by the patient’s demographic characteristics or the origin of the tumor cells. Tumor immunogenicity is influenced by tumor mutation burden, DNA mismatch repair function, antigen presentation defects, and immune checkpoint genes [22,23]. Therefore, further studies are needed to compare TIL-T levels with the molecular features and expression of immune checkpoint genes in DLBCL. Tumor immunogenicity can affect not only the prognosis of patients, but also their responsiveness to immunotherapy, such as immune checkpoint blockade or CAR-T therapy. Therefore, it is necessary to conduct additional research on the application of the TIL-T ratio criteria presented in our study (a ratio of ≥ 19% measured in the TIL-T–Max area) for immunotherapy in patients with DLBCL through digital image analysis.

As more pathology departments implement digital reviews, digital image analyses are expected to become more commonplace [24]. It is important to recognize accurate quantification facilitated by artificial intelligence for prognostic and predictive scoring as a diagnostic tool for pathologists. Several studies have shown that digital image analysis is equal to or better than manual scoring by pathologists [25,26]. The application of digital image analysis to assess TIL-T ratios in tissue specimens may help minimize inter- and intra-observer variability, thus contributing to the discovery of accurate cutoff points for TIL-T ratio analysis in the future.

This study has several limitations. First, this study was a retrospective analysis and lacked validation of the cutoff point used.

Fig. 4. (Continued from previous page)
to differentiate between high and low TIL-T levels. Second, molecular analyses of the biological factors that determine the level of TIL-T are lacking. Further studies are required to address these shortcomings, including verification of the results and cutoff values using outside cohorts. Additional subset analyses of different T-cell populations in tissue specimens and the genetic profiles of the different groups of TIL-T ratios may help understand the immunobiology of the TILs in DLBCL.

Patients with DLBCL with high TIL-T ratios showed a significantly better prognosis than those with low TIL-T ratios, and the TIL-T ratio was an independent indicator of OS. These results suggest that TIL-T may play a critical role in DLBCL disease progression and evaluating the level of TIL-T in DLBCL specimens using digital pathology software may be useful in predicting the clinical behavior and response to immunotherapy in patients with DLBCL.

Ethics Statement
All methods were performed in accordance with the Helsinki Declaration, and all protocols of this study were approved by the Institutional Review Board (IRB) of the Samsung Medical Center (IRB file number: SMC 2021-01-093-004). Formal written informed consent was not required with a waiver by the appropriate IRB.

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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Author Contributions
Conceptualization: YC, JC. Data acquisition: SEY, SJK, WSK. Data analysis: YC, JL, BH. Writing—original draft: YC. Writing—review & editing: WSK, JC. Approval of final manuscript: all authors.

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

Funding Statement
No funding to declare.

References
Follicular tumors include follicular thyroid adenomas and carcinomas, which are differentiated by the presence of capsular or vascular invasion in the resected materials or metastasis [1]. Owing to the absence of morphological differences between the tumor cells, fine-needle aspiration cytology, in which the contents of the nodule are sampled, and tissue biopsy, in which a portion of the tumor is sampled, cannot differentiate between them preoperatively [2]. Hence, many adenomas are unnecessarily resected [3,4]. If potential follicular thyroid carcinoma nodules are identified preoperatively, active surveillance of other nodules as adenomas is possible, which reduces the risk of surgical complications and the expenses incurred during medical treatment. Therefore, we aimed to identify biomarkers in the invasive subpopulation of follicular tumor cells.

Methods: We performed a spatial transcriptome analysis of a case of follicular thyroid carcinoma and examined the dynamics of CD74 expression in 36 cases.

Results: We identified a subpopulation in a region close to the invasive area, and this subpopulation expressed high levels of CD74. Immunohistochemically, CD74 was highly expressed in the invasive and peripheral areas of the tumor.

Conclusions: Although high CD74 expression has been reported in papillary and anaplastic thyroid carcinomas, it has not been analyzed in follicular thyroid carcinomas. Furthermore, the heterogeneity of CD74 expression in thyroid tumors has not yet been reported. The CD74-positive subpopulation identified in this study may be useful in predicting invasion of follicular thyroid carcinomas.

Key Words: Thyroid; Follicular carcinoma; Spatial transcriptome analysis; CD74; Capsular invasion
be determined during tissue biopsy and cytology. In this study, we aimed to identify tumor subpopulations composing invasive areas using spatial transcriptome analysis of FFPE specimens containing the invasive area of follicular thyroid carcinoma and to perform immunohistochemical studies based on these results.

**MATERIALS AND METHODS**

**Patients**

Between May 2016 and December 2022, 283 follicular thyroid carcinoma nodules were resected at the Kuma Hospital in Japan. Of these, 36 nodules (minimally invasive \( n = 18 \), encapsulated angioinvasive \( n = 8 \), widely invasive \( n = 10 \)) were extracted after excluding those with papillary-like nuclear features, poorly differentiated carcinoma components, and very small invasive areas. A nodule with an invasive area within a 5-mm-diameter circle was subjected to spatial transcriptome analysis, and all cases were subjected to immunohistochemical staining. The clinicopathological features of the enrolled 36 cases are shown in Table 1.

**Spatial transcriptome analysis**

We performed spatial transcriptome analysis using Visium CytAssist Spatial Gene Expression for FFPE (10x Genomics, Pleasanton, CA, USA). In this analysis, the whole RNA transcriptome of cells in each spot of the specialized slides was obtained from FFPE tissue sections. Each spot contained approximately 10–20 cells, and the RNA transcriptome of these cells revealed the features of RNA expression in each spot. The tissues were sectioned as described in the Visium CytAssist Spatial Gene Expression for FFPE Tissue Preparation Guide (CG000518). Sections were stained with hematoxylin and eosin (H&E), imaged, and de-cover-slipped, followed by H&E de-staining and de-crosslinking. Glass slides with tissue sections were processed using a Visium CytAssist instrument to transfer analytes to a Visium CytAssist Spatial Gene Expression slide with a 0.42 cm² capture area. Probe extension and library construction steps followed the standard Visium for the FFPE workflow. Libraries were sequenced using a DNBSEQ-G400 sequencer (BGI) (read 1: 28 bp, read 2: 100 bp). Spatial data were pre-processed and aligned using 10x Genomics Space Ranger v1.3.0 with the reference human genome GRCh38 (refdata-gex-GRCh38-2020-A) to generate raw unique molecular identifier count spot matrices. Dimensionality reduction was performed using classical principal component analysis integrated uniform manifold approximation and projection (UMAP) [15]. Unsupervised clustering of the data spots was performed using Louvain clustering [16] integrated into the BioTuring Lens with a resolution of 0.1. Further downstream analysis and gene expression visualization were conducted using the BBrowser (BioTuring, San Diego, CA, USA).

**CD74 immunohistochemical analysis**

Immunohistochemical staining was performed using 3-μm-thick FFPE specimens. Anti-CD74 (1:100, LN2, ab9514, Abcam, Cambridge, UK) and anti–thyroid transcription factor-1 (TTF-1; 1:100, SP141, ab227652, Abcam) were used as primary antibodies. Staining was performed using a Dako Autostainer Link 48+ (Dako, Carpinteria, CA, USA), according to the manufacturer’s recommendations. The expression of CD74 was assessed using a visual grading system based on staining intensity under light microscopy. High intensity (++) , low intensity (+), and no signal (−) were defined as strong, weak, and no staining, respectively. The histological index was calculated using the following formula:

\[
\text{Histological index} = 2 \times \% \text{ cells of high intensity} + 1 \times \% \text{ cells of low intensity}.
\]

The invasive area was defined as a tumor lesion invading or over the capsule or an angioinvasive lesion. The peripheral area of the tumor was defined as the tumor lesion within 1 mm of the tumor border. The tumor central area was defined as the tumor lesion > 1 mm from the border. One pathology assistant (A.S) and one pathologist (E.M) scored the data independently.

**RESULTS**

**Spatial transcriptome analysis**

Among the 36 cases of follicular thyroid carcinoma, a nodule with an invasive area within a circle of 5 mm diameter was subjected to spatial transcriptome analysis (Fig. 1A). As tissue samples in the circle could be examined in the spatial transcriptome

<table>
<thead>
<tr>
<th>Table 1. Clinicopathological features of enrolled cases</th>
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</tr>
<tr>
<td>---------------------</td>
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<td>Age (yr), mean (range)</td>
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</table>
analysis, we selected a case in which the invasive and non-invasive areas were involved in the circle. A minimally invasive case was selected for spatial transcriptome analysis. The invasive, peripheral, and central areas were shown in Fig. 1B. Louvain clustering with a resolution of 0.1 showed three clusters, and the H&E staining image merged with the three clusters (Fig. 1C). Dimensionality reduction using classical principal component analysis integrated with UMAP showed that the features of cluster 1 were distinct from those of clusters 2 and 3 (Fig. 1D). Cluster 1 was detected in the invasive area, where clusters 2 or 3 were not found (Fig. 1C, asterisk portion). The BioTuring Lens showed 10 highly expressed genes in each cluster (Table 2). CD74 was highly expressed in cluster 1 but not in clusters 2 or 3. We examined the expression of CD74 with immunohistochemistry in all 36 cases of follicular thyroid carcinoma.

CD74 immunohistochemical analyses

Immunohistochemically, the CD74 staining intensity was heterogeneous. The intensity increased in the invasive and peripheral areas of the tumors (Fig. 2A, B). Since CD74 is expressed in macrophages, double staining for CD74 and TTF-1 was performed to confirm that CD74-positive cells were follicular thyroid carcinoma cells. CD74-positive cells in the invasive and peripheral areas were TTF-1 positive, indicating that CD74

<table>
<thead>
<tr>
<th>Cluster 1</th>
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<tr>
<td>CD74</td>
<td>COL9A3</td>
<td>COL9A3</td>
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<tr>
<td>IGHG1</td>
<td>SFRP1</td>
<td>APLP2</td>
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<td>B2M</td>
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</table>

Fig. 1. Spatial transcriptomic analysis of follicular thyroid carcinoma with minimally invasive area. (A) Hematoxylin and eosin staining of examined case. (B) The invasive, peripheral, and central areas are shown. (C) Three clusters are obtained by Louvain clustering with a resolution of 0.1. Invasive area is shown with the asterisk. (D) Features of three clusters obtained by dimensionality reduction through classical principal component analysis integrated uniform manifold approximation and projection (UMAP).
positivity was detected in tumor cells located in the invasive and peripheral areas (Fig. 2C, D).

We evaluated the CD74 staining intensity in follicular thyroid carcinoma cells using a histological index (Fig. 3A). The histological index was 98.1 ± 50.6 (mean ± standard deviation) in invasive areas, 96.0 ± 48.5 in peripheral areas, and 65.8 ± 36.2 in central areas (Fig. 3B, C). Significant differences were detected between the invasive and central areas and between the peripheral and central areas (p < .01).

**DISCUSSION**

Follicular thyroid adenomas and carcinomas are collectively treated as follicular tumors. They are difficult to differentiate using fine-needle aspiration cytology [2], and the clinical management is not differentiated [3]. However, because follicular thyroid adenomas, which comprise the majority of follicular tumors, are benign, unnecessary resection is often performed in many cases. Several studies have proposed the use of various markers for distinguishing between follicular thyroid adenomas and carcinomas. Sato et al. [5] reported the utility of p53-binding protein 1 (53BP1) expression in nuclear foci, a marker reflecting the DNA damage response, to distinguish follicular thyroid carcinomas from adenomas. 53BP1 showed high sensitivity (89.3%) and specificity (83.3%), although the interpretation of results required advanced techniques. Suzuki et al. [6] attempted to differentiate between the two tumor types by determining the cell proliferation index reflecting increased nuclear DNA levels using a special flow cytometry device, LC-1000 (Sysmex Corporation, Tokyo, Japan). This method automatically calculates the index; however, a huge initial investment is required. An easier method to incorporate is immunohistochemistry, and antibodies such as ki-67, bax, secreted protein acidic and rich in cysteine, HBME1, Rac1, Galectin-3, and CD61 have been investigated, but none of them is currently in clinical use [7-12].

RNA sequences of microdissected samples are useful for identifying molecular markers; however, they only provide information on the bulk expression of tumor cells in the microdissected area. The most precise investigation of RNA expression is a single-cell RNA sequence; however, this method loses the spatial

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**Fig. 2.** Immunohistochemical analyses of CD74 in follicular thyroid carcinoma. (A) Hematoxylin and eosin staining. (B) Immunohistochemical staining using anti-CD74 antibody. (C) Double immunohistochemical staining using anti-CD74 (brown) and anti-thyroid transcription factor-1 (red) antibodies. (D) High power field of (C).
information of each individual cell. Spatial transcriptome analysis is a new technique that enables whole-transcriptome analysis without microdissection of FFPE specimens while maintaining morphological information; it has been investigated in a variety of tissues \([13,14]\). In this study, we performed spatial transcriptome analysis using follicular thyroid carcinoma and identified a subpopulation in the invasive area that was found to express high levels of CD74.

CD74 is known to play an important role in antigen presentation by mediating the construction of major histocompatibility complex II complexes and intracellular trafficking \([17]\). CD74 has also been reported to be upregulated in malignant tumors and involved in increased growth and metastatic potential \([18-28]\). In a study on urothelial bladder carcinomas by Choi et al. \([18]\), urothelial bladder carcinomas with high CD74 expression were characterized by older age, high World Health Organization grade, and advanced stages of TNM classification. In a study on gastrointestinal carcinomas, Gold et al. \([26]\) found that the expression of CD74 in gastrointestinal carcinomas was significantly greater than that in their normal tissue counterparts (\(p < .001\) or lower). With regard to thyroid carcinomas, Varinelli et al. \([27]\) and Cheng et al. \([28]\) reported that the CD74 axis plays an important role in the biology of aggressive cases in papillary and anaplastic thyroid carcinomas. In these two studies \([27,28]\), CD74 staining was detected in all tumor cells of aggressive cases, and no heterogeneous staining was reported. To our knowledge, the present study is the first report of CD74 staining in follicular thyroid carcinomas and the heterogeneous staining intensity of CD74 was detected for the first time, suggesting the presence of an invasive subpopulation of follicular tumor cells. In a study by Cheng et al. \([28]\), treatment with anti-CD74 antibody in papillary thyroid carcinoma cell lines inhibited cell growth, colony formation, cell migration and invasion, and vascular endothelial growth factor secretion. Our results suggested that CD74 might play a role in cell invasion and might be a novel therapeutic target for follicular thyroid carcinomas, such as papillary thyroid carcinoma.

Our results demonstrated a significantly higher staining score in the invasive area than in the central area (\(p < .01\)), suggesting that the CD74-positive subpopulation may be used to predict the invasion of follicular tumors. Although there was no significant difference between the invasive and peripheral areas just below the capsule (\(p = .429\)), Gold et al. \([26]\) stated that precursor lesions might express the same or higher CD74 levels as the respective cancers, as the activation of survival pathways was particularly important in the early stages of tumorigenesis. It was possible that the positive cells observed immediately below the capsule in our study were near invasion. Although this study was performed on resected material, we expect that immunocytochemical staining for CD74 will be performed in the future on nodules suspected of having follicular tumors on cytological examination or biopsy tissue specimens to determine the possibility of invasion before surgery, which will contribute to reducing unnecessary resection of nodules with a low possibility of invasion.

In this study, we only included follicular thyroid carcinomas with a clear invasion. Future studies are required to examine CD74...
Identifying invasive follicular tumour cells

immunostaining in patients with follicular tumors of uncertain malignant potential [1] with unclear histological status to identify whether they are follicular thyroid adenomas or carcinomas and prospectively observe their prognosis. The correlation between the staining results and prognosis provided a better confirmation of the relationship between CD74 immunostaining and invasion.

Ethics Statement
The study protocol was reviewed and approved by the Institutional Review Board of Kuma Hospital (no. 20230112-2, 12/01/2023) and Osaka University Clinical Research Review Committee (no. 22425, 17/02/2023). This study complied with the 1964 Declaration of Helsinki and its later amendments. All study participants provided 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.

Author Contributions
Conceptualization: SN, EM, AS. Data curation: SN, EM. Formal analysis: SN, AS. Investigation: SN, AS, MK. Methodology: SN, ST, DM, DO, MK. Project administration: SN, EM. Resources: EM. Supervision: SN, EM. Writing—original draft: AS, EM. Writing—review & editing: SN, ST, DM, DO, MH, EM. Approval of the final manuscript: all authors.

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

Funding Statement
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Acknowledgments
We would like to thank Editage (www.editage.jp) for English language editing.

References
3. Haugen BR, Alexander EK, Bible KC, et al. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid 2016; 26: 1-133.
19. Hong WC, Lee DE, Kang HW, et al. CD74 promotes a pro-inflam-
matory tumor microenvironment by inducing S100A8 and S100A9 secretion in pancreatic cancer. Int J Mol Sci 2023; 24: 12993.
Neuroblastomas (NB) are tumors deriving from the sympathoadrenal lineage - one of the neural crest derivatives. NB represents the most prevalent extracranial solid tumor in children and accounts for 12% of all childhood cancer deaths [1]. The presence of anaplastic lymphoma kinase (ALK) mutations in NB has been published since 2008 [2]. Approximately 8% to 10% of NB have ALK mutations, and about 25% have ALK amplification or overexpression of the ALK protein [3]. Many studies have shown that the ALK gene is a significant oncogene in NB, and ALK mutations have been identified in association with familial and sporadic NB [2]. Besides, ALK is a consummate tumor antigen for targeted inhibition, due to its frequent expression in NB and restricted distribution in normal tissue [4]. These results open the door to new therapeutic strategies for treating NB with ALK inhibitors.

Several research groups have reported that ALK overexpression in NB is an adverse prognostic factor, however, the relations between ALK protein expression and clinical and histopathological features of NB are still controversial [2,5-7]. There are many methods to evaluate ALK in NB, in which immunohistochemistry (IHC) is available and capable of routine application. The ALK D5F3 antibody showed the highest sensitivity compared to other clones such as ALK 1, and 5A4. The ALK D5F3 antibody is likely the most appropriate antibody for determining ALK protein expression levels in NB, which may be used to predict patient prognosis [8,9].

**Background:** Anaplastic lymphoma kinase (ALK) mutations have been identified as a prominent cause of some familial and sporadic neuroblastoma (NB). ALK expression in NB and its relationship with clinical and histopathological features remains controversial. This study investigated ALK expression and its potential relations with these features in NB. **Methods:** Ninety cases of NB at the Department of Pathology, University of Medicine and Pharmacy at Ho Chi Minh City, Viet Nam from 01/01/2018 to 12/31/2021, were immunohistochemically stained with ALK (D5F3) antibody. The ALK expression and its relations with some clinical and histopathological features were investigated. **Results:** The rate of ALK expression in NB was 91.1%. High ALK expression (over 50% of tumor cells were positive with moderate-strong intensity) accounted for 65.6%, and low ALK expression accounted for 34.4%. All the MYCN-amplified NB patients had ALK immunohistochemistry positivity, most cases had high ALK protein expression. The undifferentiated subtype of NB had a lower ALK-positive rate than the poorly differentiated and differentiated subtype. The percentages of ALK positivity were significantly higher in more differentiated histological types of NB (p = .024). There was no relation between ALK expression and: age group, sex, primary tumor location, tumor stage, MYCN status, clinical risk, Mitotic-Karyorrhectic Index, prognostic group, necrosis, and calcification. **Conclusions:** ALK was highly expressed in NB. ALK expression was not related to several clinical and histopathological features. More studies are needed to elucidate the association between ALK expression and ALK gene status and to investigate disease progression, especially the oncogenesis of ALK-positive NB.

**Key Words:** Neuroblastoma; Anaplastic lymphoma kinase
In this study, ALK protein expression was studied to investigate potential relations with some clinical and pathological features of NB.

MATERIALS AND METHODS

Patients and specimens

Patients who were histologically diagnosed with NB (Schwannian stroma-poor) (based on International Neuroblastoma Pathology Classification [INPC]) at the Department of Pathology, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam from January 2018 to December 2021 were enrolled in this study. All tumor specimens were obtained from the primary or metastatic tumor through surgery or biopsy before starting chemotherapy. The clinical parameters, including age at diagnosis, gender, tumor stages (based on International Neuroblastoma Risk Group Staging System [INRGSS]), primary tumor sites were collected. MYCN status (by fluorescence in situ hybridization [FISH]) was retrospectively inquired through record data from the Center for Molecular Biomedicine, University of Medicine and Pharmacy at Ho Chi Minh City. Hematoxylin a eosin slides were assessed for degree of differentiation, Mitotic-Karyorrhectic Index (MKI), and prognostic groups (according to INPC). In addition, tumor necrosis and calcification will also be noted.

Formalin-fixed, paraffin-embedded tumor tissue collected during surgery or biopsy was used. For surgical specimens, a representative area of tumor tissue will be submitted for tissue microarray, and whole biopsied specimens will be collected for IHC staining.

IHC staining

IHC staining was performed on 4-μm-thick sections using VENTANA anti-ALK (clone D5F3, Ventana Medical Systems, Tucson, AZ, USA) rabbit monoclonal primary antibody was used in this study. The IHC staining process was fully automated using a BenchMark XT automated slide stainer (Ventana Medical Systems).

Cytoplasmic and membrane staining with any ratio was considered positive. Negative when absolutely no tumor cells were stained. ALK IHC staining was scored based on the percentage of antibody-reactive neoplastic cells and the intensity as follows: “0” (negative, no stained cells), “1+” (weak expression, < 20% of cells stained), “2+” (heterogeneous weak-moderate expression, around 20%–50% of cells stained), “3+” (heterogeneous moderate-strong expression, > 50% of cell stained), and “4+” (> 75%, strong expression).

Cases with ALK IHC score 3+, and 4+ are considered to have high ALK protein expression (i.e., more than 50% of tumor cells are stained with moderate-strong intensity). The remaining cases were considered to have low ALK protein expression (IHC ALK score 0, 1+, 2+).

Statistical analysis

Pearson’s χ² test (or Fisher’s exact test if the sample size was small) was used to evaluate relationship between pairs of categorical variables. All statistical analyses were performed using STATA ver. 14.2 (Stata Corp., College Station, TX, USA). p-value < .05 were statistically significant.

RESULTS

Patient characteristics

A total of 90 patients were enrolled in this study. The clinical and pathological characteristics of the patients are summarized in Table 1. The median age of patients was 24 months old (15 days old–14 years old), and the group under 18 months old accounted for the majority. The male/female ratio is 1.3:1. In 80 cases tested for FISH MYCN, 11.3% had MYCN amplification. More than 80% of NB were poorly differentiated subtype, unfavorable histology group accounted for the majority with 57.8% (Table 1).

ALK protein expression

ALK was positive in 91.1% of cases (82/90 cases), in which high ALK expression (3+, 4+) accounted for 65.6% (59/90 cases). The ALK IHC expression is summarized in Table 2.

ALK IHC staining was observed in the cytoplasm or membrane of NB cells and was negative for lymphocytes, endothelium, and normal adrenal gland (Fig. 1). Different intensity of ALK IHC was shown in Fig. 2.

The relation between ALK expression and some clinical and pathological features

Our study showed all cases of differentiating NB were ALK-positive (Table 3). There was significant relation of ALK positivity with NB histological types (undifferentiated NB [9/13, 69.2%] vs. poorly differentiated NB [69/73, 94.5%] vs. differentiating NB [4/4, 100%; p = .024]). All cases with MYCN amplification were positive for ALK IHC, in which high ALK expression accounted for 7/9 cases (77.8%). No other relations between ALK expression and clinical and pathological features were observed (Table 3).
DISCUSSION

Until now, there is no consensus method for evaluating ALK IHC staining in NB, most of the studies were based on the percentage of ALK antibody-reactive neoplastic cells and modified evaluation methods depending on the research groups [2,5,8, 10-13]. Several previous studies reported the relation between high ALK expression (> 50% of tumor cells) and poor clinical outcome [2,5,13], so a positive threshold of more than 50% of stained tumor cells was applied in many studies [6,7]. In this study, we applied the modified assessment method according to Passoni et al. [2], and investigated the relations between high/low ALK protein expression with some clinical and histopathological factors. Our study showed that the ALK expression rate was 91.1%, similar to many other studies [2,5,9,10]. ALK protein is a single-chain transmembrane protein comprised of three regions: extracellular domain, transmembrane region, and intracellular domain [4], so ALK IHC showed cytoplasmic/membranous expression. ALK protein is expressed on the surface of most NB cells, and its expression is uncommon in normal tissue [4,9]. This makes ALK an ideal target for cancer therapy, whether the NBs exhibit ALK mutations [4,9].

In this study, ALK expression was not related to clinical features including age, sex, stage, or MYCN status. A previous study reported that the incidence of ALK expression increased correspondingly with more advanced tumor stages (p = .001) [7]. In another study by Passoni et al. [2], ALK protein expression was significantly up-regulated in advanced/metastatic NB, however, our study showed that ALK expression was not related to the disease stage [2]. This discrepancy may be attributed to differences in the staging system used in the two studies (INRGSS vs. International Neuroblastoma Staging System [INSS]), and the ALK antibody clone (D5F3 vs. 5A4, ALK1, SP8, each antibody binds to a different epitope, which may explain why they have different sensitivities [14]).

Our study revealed that all cases with MYCN amplification were positive for ALK IHC (Table 3), in which high ALK expression accounted for 7/9 cases (77.8%). Similar to our results, Lee et al. evaluated ALK expression on 70 NBs and reported that all seven cases with MYCN amplification or gain were more likely to be ALK-positive than tumors with normal MYCN status (p < .05) [6]. Yan et al. [8] found a strong relationship between ALK D5F3 IHC and the number of copies of the MYCN gene on NB. Schonherr et al. [15] reported that ALK activity was

<table>
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<th>No. (%)</th>
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<tr>
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<td>43 (47.8)</td>
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<td>68 (75.6)</td>
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INRGSS, International Neuroblastoma Risk Group Staging System; MKI, Mitotic-Karyorrhectic Index; INPC, International Neuroblastoma Pathology Classification.

<table>
<thead>
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<th>ALK IHC score</th>
<th>No. (%)</th>
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<td>Low expression</td>
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<td>8 (8.9)</td>
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<tr>
<td>1+</td>
<td>10 (11.1)</td>
</tr>
<tr>
<td>2+</td>
<td>13 (14.4)</td>
</tr>
<tr>
<td>High expression</td>
<td></td>
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<tr>
<td>3+</td>
<td>13 (14.4)</td>
</tr>
<tr>
<td>4+</td>
<td>46 (51.1)</td>
</tr>
<tr>
<td>Total</td>
<td>90 (100)</td>
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</table>

ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry.
very important in MYCN transcription initiation, and MYCN gene transcription was eliminated by using a specific ALK inhibitor. From our observations combined with several reports that high ALK expression in NB is predictive of treatment response to ALK inhibitors [2,9], we speculate that MYCN-amplified NB patients may benefit from ALK inhibitors treatment if high levels of ALK expression are present.

We noted higher percentages of ALK positivity in more differentiated histological types of NB (p = .024). However, there was no statistically significant correlation between ALK IHC score and NB histological types (p = .143), similar to another study [10]. ALK expression is found in neural crest cells during early development, and the involvement of ALK activation in neural crest cell migration and proliferation has been shown [16]. However, how ALK helps neural crest cells to develop in humans is poorly understood [17]. In our study, up to 4/13 cases (30.8%) of undifferentiated NB were negative for ALK IHC. As ALK protein expression was not associated with ALK mutations in most cases (half or more of NBs have strong ALK protein expression, but only about 10% of NBs harbor ALK aberrations) [2,5,8], so further molecular biology tests are needed to determine if these ALK IHC-negative cases have the ALK gene mutations. The D5F3 IHC test is highly concordance with ALK-FISH and approved by the U.S. Food and Drug Administration for detecting ALK rearrangements in non-small cell lung carcinoma; however, the discrepancy between these two tests has also been reported [18]. In addition, in the group of pulmonary neuroendocrine tumors, many studies have shown that there is a mismatch between ALK protein expression and ALK alterations [19-22]. Nakamura et al. [19] indicated that the immunopositivity is probably of a wild-type ALK, which may be due to epigenetic regulation or protein overstabilization. Although there are no ALK alterations detected by FISH, cases of lung cancer overexpressing ALK protein that respond positively to ALK inhibitors were announced [23,24]. Similar to NB, ALK-positive tumors may still respond to ALK inhibitor drugs even if tumors

Fig. 1. (A) Neuroblastoma (left side) and normal adrenal gland (right side). (B) Strong immunohistochemistry staining of anaplastic lymphoma kinase (ALK) in tumor cells (left side), no ALK staining is found in normal adrenal tissue (right side).

Fig. 2. (A) Immunohistochemistry (IHC) staining for anaplastic lymphoma kinase (ALK) in neuroblastoma (NB) showed strong, homogeneous cytoplasmic or membraned staining. (B) Moderate ALK IHC staining in NB. (C) Weak, heterogeneous ALK IHC staining in NB.
lack ALK mutations [5,9], therefore, ALK-negative undifferentiated NB cases may be an indicator of unresponsiveness to ALK inhibitors. Until now, many generations of ALK inhibitors have been created and included in research. Overcoming the limitations of the pioneering ALK inhibitor crizotinib, several novel designed generations of ALK inhibitors and the combinatory therapy with either pathway inhibitors or other agents against other targets are also of interest [25,26]. The definitive efficacy of ALK inhibitors in NB remains to be elucidated upon the conclusion of ongoing clinical trials within the next few years [25].

In summary, this study highlights the characteristics of ALK expression in NB by applying both approaches to evaluating ALK expression in NB and clarifies the reason for the difference in the results of previous studies. ALK expression was not related to several clinical and histopathological features. Our study revealed a significant relation between ALK positivity with NB histological types, and all cases with MYCN-amplified NB patients may benefit from ALK inhibitors. Since the role of ALK in neural crest development is still unclear, further studies are needed to elucidate the association between ALK expression and ALK gene status and to investigate disease progression, especially the oncogenesis of ALK-positive NB.

**Ethics Statement**

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Bio-

### Table 3. The relation between ALK expression and some clinical and pathological features

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of cases</th>
<th>IHC ALK-positive</th>
<th>IHC ALK-negative</th>
<th>p-value</th>
<th>Low ALK expression</th>
<th>High ALK expression</th>
<th>p-value</th>
</tr>
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<tr>
<td>Age (mo)</td>
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<td></td>
<td></td>
<td>.704a</td>
<td>14 (32.6)</td>
<td>29 (67.4)</td>
<td>.928a</td>
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<td>&lt;18</td>
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<td>40 (93.0)</td>
<td>3 (7.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–60</td>
<td>31</td>
<td>28 (90.3)</td>
<td>3 (9.7)</td>
<td>11 (35.5)</td>
<td>20 (64.5)</td>
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<tr>
<td>≥60</td>
<td>16</td>
<td>14 (87.5)</td>
<td>2 (12.5)</td>
<td>6 (37.5)</td>
<td>10 (62.5)</td>
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<td>.723a</td>
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<td>.124a</td>
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<tr>
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<td>51</td>
<td>47 (92.2)</td>
<td>4 (7.8)</td>
<td>21 (41.2)</td>
<td>30 (58.8)</td>
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<tr>
<td>Female</td>
<td>39</td>
<td>35 (89.7)</td>
<td>4 (10.3)</td>
<td>10 (25.6)</td>
<td>29 (74.4)</td>
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<td>Stage (INRGSS)</td>
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<tr>
<td>L1</td>
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<td>23 (92.0)</td>
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<td>11 (42.3)</td>
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<tr>
<td>M</td>
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<td>MYCN</td>
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<td>2 (22.2)</td>
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<tr>
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<td></td>
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<td></td>
<td>.143a</td>
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<tr>
<td>Poorly differentiation</td>
<td>73</td>
<td>69 (94.5)</td>
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<td>22 (30.1)</td>
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<tr>
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<td>2 (50.0)</td>
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<td>MKI</td>
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<td></td>
<td>.682a</td>
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<td></td>
<td>.230a</td>
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<tr>
<td>Low</td>
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<td>45 (88.2)</td>
<td>6 (11.8)</td>
<td>21 (41.2)</td>
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<td>16 (80.0)</td>
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<td>Prognostic group</td>
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<td>&gt; .99a</td>
<td>14 (36.8)</td>
<td>24 (63.2)</td>
<td>.682a</td>
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<tr>
<td>Favorable</td>
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<tr>
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<td>52</td>
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<td>5 (9.6)</td>
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<td>.735a</td>
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<tr>
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<td>27</td>
<td>23 (85.2)</td>
<td>4 (14.8)</td>
<td>10 (37.0)</td>
<td>17 (63.0)</td>
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</tr>
<tr>
<td>No</td>
<td>63</td>
<td>59 (93.7)</td>
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<td>21 (33.3)</td>
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<td>7 (10.3)</td>
<td>21 (30.9)</td>
<td>47 (69.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as number (%).

ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry; INRGSS, International Neuroblastoma Risk Group Staging System; MKI, Mitotic-Karyorrhectic Index.

*Fisher’s exact test; **χ² test.

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medical Research of University of Medicine and Pharmacy at Ho Chi Minh City (IRB No. 174/HDDD-DHYD; on February 21, 2022). Informed consent was obtained from all individual participants included in the study.

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: TDA, TQN, DQN. Data curation: TDA, TQN, TLT. Formal analysis: TDA, TQN. Funding acquisition: TDA, TLT. Investigation: TQA, TQN, TLT. Methodology: TQA, TQN. Supervision: DQN. Writing—original draft: TLN, TQA. Writing—review & editing: TDAP, TQN, DQN. 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
Primary leiomyosarcoma of the bone: a case report

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Primary leiomyosarcoma of the bone is rare. Histologically, it resembles leiomyosarcoma of soft tissue. Given the rarity of this entity, its diagnosis should be made only after clinical studies and workup have excluded metastasis from other sites. Herein, we describe an additional case of primary bone leiomyosarcoma. We report a 32-year-old female patient, who presented with right knee pain and was found to have a right distal femur mass by imaging studies. Biopsy showed a neoplasm composed of fascicles of spindle cells, arranged in different patterns, with significant pleomorphism. The tumor cells were positive for smooth muscle actin, focally positive for desmin and H-caldesmon. No other masses in the body were detected by imaging studies. The diagnosis of leiomyosarcoma of the bone was rendered. Given the broad diagnostic differential of primary bone leiomyosarcoma, it is important to be aware of this rare bone tumor phenotype and of its histomorphologic and immunohistochemical features for an accurate diagnosis.

Key Words: Bone; Sarcoma; Leiomyosarcoma; Case report

CASE REPORT

A 32-year-old, medically-free female patient presented with right knee pain of 10-day duration. The pain worsened at night and with physical activities. Physical examination was unremarkable. X-ray revealed a right distal femur intraosseous metaphyseal lytic lesion with cortical thinning and soft tissue extension. Magnetic resonance imaging showed a well-defined central destructive lesion, completely replacing the medullary cavity of the distal third of the right femoral shaft for a 10 cm span in craniocaudal dimension, reaching the anterior aspect of both femoral condyles and the posterior aspect of the intercondylar fossa with multiple areas of cortical bone destruction (Fig. 1). The patient underwent a core needle biopsy of the right distal femur lesion.

Histopathologic findings

Gross examination revealed multiple tan-pink irregular friable focally hemorrhagic soft tissue fragments, measuring 2.5 × 1.7 × 0.4 cm in aggregate. Microscopic examination demonstrated fascicles of spindle and epithelioid cells with plump, blunt-ended nuclei, occasional subnuclear vacuoles, and moderate brightly
 eosinophilic cytoplasm. There was significant pleomorphism in the form of hyperchromasia, high nucleus-to-cytoplasmic ratio, and irregular nuclear contours, arranged in intersecting, storiform, and haphazard patterns (Fig. 2A, B). Other cells showed vesicular nuclei and prominent nucleoli. Multinucleated giant cells were also identified. The background was sclerotic. Fat lobules infiltrated by the atypical cells were also seen (Fig. 2C). Mitotic activity was 5 per 10 high-power fields.

Immunohistochemical findings

On immunohistochemical stains, performed by machine, the tumor cells were positive for SMA (ready-to-use, Dako, Glostrup, Denmark) “shown in Fig. 2D”, focally positive for desmin (ready-to-use, Dako) (Fig. 2E), H-caldesmon (ready-to-use, Dako) (Fig. 2F), CD10 (ready-to-use, Dako), CD68 (ready-to-use, Dako) and SATB2 (prediluted, Cell Marque, Rocklin, CA, USA), and negative for cyto keratin (CK) AE1/AE3 (ready-to-use, Dako), CK MNF 116 (1:50, Dako), CDK4 (prediluted, Cell Marque), MDM2 (prediluted, Cell Marque), Melan-A (ready-to-use, Dako), S100 (ready-to-use, Dako), SOX-10 (prediluted, Cell Marque), estrogen receptor (ER; ready-to-use, Dako) (Fig. 2G), progesterone receptor (PR; ready-to-use, Dako), CD31 (ready-to-use, Dako), CD34 (ready-to-use, Dako), and myogenin (ready-to-use, Dako). S100 (ready-to-use, Dako) was positive in the adipocytes. Ki-67 (ready-to-use, Dako) proliferation index was high.

Final diagnosis and clinical course

The diagnosis of high-grade sarcoma, most consistent with leiomyosarcoma of the bone, was rendered. The patient had no previous gynecological history. Imaging studies revealed no uterine masses (Fig. 3) or soft tissue masses, and no lung or bony metastatic lesions. The patient underwent wide excision of the right distal femur. The gross examination of the resected specimen revealed an intramedullary irregular tan-white ill-defined infiltrative lesion measuring 9.2×6.3×3.2 cm. The final diagnosis on the excision specimen concurred with the biopsy diagnosis. The TNM stage was pT2 N0. The patient will be followed up by the oncology team on a regular basis.

DISCUSSION

Leiomyosarcomas are aggressive malignant soft tissue tumors arising from smooth muscle cells. They are common among soft tissue sarcomas, comprising approximately 25% of all sarcomas [8]. Primary bone leiomyosarcoma is a rare tumor, accounting for less than 0.7% of primary malignant bone neoplasms [1]. It was first described by Evans and Sanerkin in 1965 [5], and is slightly more common in males, with its incidence peaking at the fifth decade of life [2]. It mostly occurs at the distal femur followed by the proximal tibia and proximal humerus [3]. A small number of cases have been found to be associated with previous radiation therapy or EBV infection [2]. The most common symptom of primary bone leiomyosarcoma is pain, followed by the presence of a palpable mass and a pathologic fracture [9]. On radiographic examination, it usually presents as an intraosseous aggressive lytic lesion with a permeative pattern of osseous destruction [10]. The outline of low-grade lesions shows regular cortical destruction and is demarcated by a sclerotic rim whereas high-grade lesions have an ill-defined outline and lack a sclerotic rim. On gross examination, the lesions vary in size and have gray-tan creamy appearance with foci of necrosis and cystic degeneration [9]. Histologically, it resembles leiomyosarcoma of soft tissue with intersecting fascicles and bundles of pleomorphic spindle cells. The cells are characterized by eosinophilic cytoplasm and cigar-shaped nuclei with blunted ends. High-grade leiomyosarcomas are associated with marked pleomorphism, necrosis, and significant mitotic activity. Immunohistochemical findings demonstrate diffuse positivity for SMA, desmin, and H-caldesmon [2].

The cellular origin of primary bone leiomyosarcoma remains controversial. A vascular smooth muscle cell origin has been sug-
gested due to the high vascularity of some of these lesions, while others have suggested that it can originate from multipotential mesenchymal stem cells capable of undergoing smooth muscle differentiation [11,12].

Given the rarity of primary bone leiomyosarcoma, metastasis from other sites should always be carefully excluded through clinical examination and imaging studies. In female patients, positivity for ER or PR immunostains hints toward a primary uterine origin [13]. In our case, the patient was medically free and had no previous history of leiomyosarcoma and her physical examination and pan–computed tomography findings were unremarkable. Based on this, the diagnosis of primary bone leiomyosarcoma was rendered.

In 1997, a case series on thirty-three patients with primary bone osteosarcoma was conducted [9]. In this study, they found that the patient’s age at diagnosis ranged from 13 to 77 years (average 44.4) with a male-to-female ratio of 1:1. The long bones were mostly affected, with distal femur and proximal tibia predominantly involved. Post-radiation bone leiomyosarcoma occurred in 15% of the patients, and seventy-five percent of the cases were high-grade with worse prognosis [9]. A recent case series was conducted on twenty-two patients with primary bone leiomyosarcoma. Researchers found that the patient’s age at diagnosis ranged from 17 to 73 years (average 45.5), and there was a slight male predominance. The lower limb was the most affected site. In this study, they reported 90.9% of the cases as high-grade, with distant metastasis reported in 40.9% of the patients and an overall survival rate of 59.1% in 5 years [14].

Fig. 2. Microscopic and immunohistochemical features of primary bone leiomyosarcoma. (A) Photomicrograph shows fascicles of pleomorphic spindle and epithelioid cells arranged in fascicular, storiform, and haphazard patterns. (B) High-power view demonstrates cellular pleomorphism in the form of hyperchromasia, high nucleus-to-cytoplasmic ratios, and irregular nuclear contours. Some cells show vesicular nuclei and prominent nucleoli. (C) Photomicrograph shows fat lobules infiltrated by tumor cells. (D) The tumor cells are positive for smooth muscle actin. (E) The tumor cells show focal reactivity for Desmin. (F) The tumor cells show focal reactivity for H-caldesmon. (G) The tumor cells are negative for estrogen receptor.
Compared to the previous studies, our case involves a 32-year-old female patient with no previous medical history or exposure to radiation. The tumor was located at the right distal femur. The patient has not developed local recurrence or metastasis since the time of diagnosis and treatment.

The main differential diagnoses of primary bone leiomyosarcoma include osteosarcoma, chondrosarcoma, myxofibrosarcoma, synovial sarcoma, malignant peripheral nerve sheath tumor (MPNST), fibrosarcoma, undifferentiated pleomorphic sarcoma and metastatic carcinoma [4]. The lack of malignant osteoid can help in excluding osteosarcoma, though this may be difficult in fibroblastic osteosarcomas since osteoid is focally present in such cases. Osteosarcomas may express desmin which will immunophenotypically overlap with leiomyosarcomas. Therefore, osteosarcomas should be carefully excluded after good radiological investigation and extensive sampling [4]. The lack of malignant cartilage production rules out chondrosarcoma. Synovial sarcoma usually demonstrates monotonous spindled cells with scant amphophilic cytoplasm, has a specific chromosomal translocation t(X;18) (p11;q11) and usually expresses TLE1, epithelial membrane antigen, and keratins. Features favoring leiomyosarcoma against MPNST include blunt-ended cigar-shaped nuclei with bright eosinophilic cytoplasm, positivity for SMA, H-caldesmon, and desmin. The diagnosis of fibrosarcoma and undifferentiated pleomorphic sarcoma is made by exclusion of other entities as they lack specific immunohistochemical and genetic markers [2,4]. Metastatic carcinomas express epithelial markers and lack myogenic markers. Lack of well-differentiated liposarcomatous component, MDM2 and CDK4 immunostains expression, and 12q13-15 amplification rules out dedifferentiated liposarcoma.

Data regarding the treatment modalities and prognosis of primary bone leiomyosarcoma is still limited due to its low prevalence. In one study, researchers reported that half of the patients with primary bone leiomyosarcoma developed metastases within one year of diagnosis [15].

In summary, we describe an additional case of primary bone leiomyosarcoma, which adds on more data about this rare category of bone tumors. In our case, the patient was medically free, had no previous history of leiomyosarcoma and no previous gynecological history, and had no uterine or soft tissue masses, along with the negative results of ER and PR in the tumor, which excludes the possibility of metastatic leiomyosarcoma from the uterus. Given the broad diagnostic differential of primary bone leiomyosarcoma, it is important to be aware of this rare bone tumor phenotype and its histomorphological and immunohistochemical features for accurate diagnosis.

**Ethics Statement**
The Institutional Review Board at Hamad Medical Corporation approved publication of this article under the number (MRC-04-22-670). Informed consent from the participant has been waived by 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.

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

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**References**
Dear Editor,

We read with great interest the article “A stepwise approach to fine needle aspiration cytology of lymph nodes” by Chong et al. [1], recently published in *J Pathol Transl Med*. The Authors proposed a morphological stepwise algorithm, separate but complementary to the Sydney system, for the cytological diagnosis of lymphadenopathies [2]. In the proposed algorithm the basic diagnostic criteria are structured in a stepwise approach for cytological diagnosis of adenopathy. Regarding the diagnostic classification the Authors basically agree with the Sydney system [2] but they state that “…the epithelial or lymphomatous nature of the lesion is not clearly separated into different categories and is not emphasized due to the ambiguity of the lymph node features on FNAC. In other words, even though a diagnosis was made on a certain case using the Sydney system, it is still unclear whether the case is lymphoid or metastatic.” We disagree with this observation because when a case is atypical, suspicious, or malignant, the Sydney System strongly encourages to report the nature (lymphoid/metastatic) of the nosological entity. This is also in keeping with clinical practice, since no hematopathologist would ever omit such information from a report. Even in cases in which the cytomorphology is insufficient to distinguish between a lymphoid or metastatic malignancy, a comment will be added to state this.

One additional important point is that the stepwise system proposed by the authors is purely morphological and does not consider ancillary techniques. While the two-level structure of the Sydney System was intentionally conceived to be applicable even in settings where ancillary testing is unavailable, it is important to note that cytopathologists should strive to perform ancillary techniques whenever possible, for three reasons. First, it helps limit atypical and suspicious cases and categorize them in a definite category instead (benign or malignant) [3]. Second, it helps to further subclassify the nosological process responsible for the lymphadenopathy (e.g., “malignant, lymphoma” can become “malignant, follicular B-cell lymphoma” after flow cytometry; “malignant, metastasis” can become “malignant, breast cancer metastasis” after immunocytochemistry; and “benign, granulomatous” can become “benign, tuberculous lymphadenopathy” after polymerase chain reaction). Third, in some cases a fully actionable diagnosis, including prognostic and predictive biomarkers, can be performed on lymph node fine needle aspiration cytology (LN-FNAC). For example, after harvesting non–small-cell lung cancer material from a nodal metastasis, it is possible to employ cytoclastic material to guide targeted therapy [4].

In fact, the relatively high risk of malignancy (ROM) registered for the category atypical (atypical, undermined significance—atypical lymphoid uncertain significance, AUS-ALUS) by Gupta et al. [5] was the highest among similar series which applied the Sydney System to LN-FNAC [3], mainly because no ancillary techniques were utilized. This high ROM was foreseen by the authors of the Sydney System because some differential diagnoses, such as that between florid reactive hyperplasia and follicular lymphoma or other low-grade non-Hodgkin lymphoma, can be difficult or almost impossible using only cytomorphological criteria (Fig. 1). This differential diagnosis by FNAC, other than in lymphadenopathies, is crucial in extranodal lymphoproliferative processes in which diagnostic excision or invasive biopsies might be useless or even harmful [6,7]. Focusing on the proposed algorithm, we do not believe that only cytomorphological criteria will reduce the incidence of this diagnostic category and its ROM. On the other hand, ancillary techniques (flow cytometry, immunohistochemistry or molecular testing) combined

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**LETTER TO THE EDITOR**

**Comment on “A stepwise approach to fine needle aspiration cytology of lymph nodes”**

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with clinical data and cytological features, can discriminate these entities in many cases. For this reason, the Sydney System [2] strongly suggests the application of ancillary techniques in these cases because cytological criteria alone cannot discriminate between entities included with similar cytological patterns. Moreover, repetition of LN-FNAC in AUS-ALUS cases, after six-eight weeks may show dramatically changed cytological features in case of reactive hyperplasia. The authors [1] also state that “the L3 category, which includes AUS and ALUS, is a good example. It is often quite challenging to discriminate a certain lesion as lymphoid or epithelial in origin” and that the Sydney System includes “cases in which the FNAC features favor certain diseases, the cases should be described specifically as “AUS, favor poorly differentiated carcinoma,” This sentence is not reported in the Sydney system text in which suspicious lymphoma and metastases are included in the category suspicious, which has a higher ROM and different clinical implications than the atypical category. Regarding some specific entities (progressive transformation of germinal centers, Kikuchi-Fujimoto disease, Rosai-Dorfman disease, or others) there is relatively little FNAC experience on their cytological features and few case reports or small series are available in the literature to foresee their diagnostic category (AUS-ALUS or suspicious) and corresponding ROM. We are aware that the ROM of diagnostic categories largely depends on technical issues, different clinical contexts, local epidemiology, and on the reproducibility of diagnostic criteria. For this purpose, a recent experience on LN-FNAC digital slides has been performed producing quite encouraging results [8]. In conclusion we think that the algorithm proposed by Chong et al. [1] matches with the Sydney system which will be reproduced in five diagnostic categories and two diagnostic levels also by the near to be published WHO Reporting System for Lymph Node, Spleen, and Thymus Cytopathology.

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

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References
Response to comment on “A stepwise approach to fine needle aspiration cytology of lymph nodes”

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To the Editor,

We read Caputo et al.’s letter [1] regarding our recently published review, “A Stepwise Approach to Fine Needle Aspiration Cytology of Lymph Nodes,” with great interest and we really appreciate their keen observation and critique to our review [2]. Their critique of our publication focuses on three primary issues: (1) The Sydney system’s strong recommendation for reporting the nosologic entity’s nature (lymphoid/metastatic) [3]. (2) Our proposed stepwise system’s exclusive focus on morphology, overlooking ancillary techniques like flow cytometry and polymerase chain reaction. (3) The heightened risk of malignancy in the AUS-ALUS (atypical, undermined significance–atypical lymphoid uncertain significance) category, as highlighted in Gupta et al.’s study [4], attributed mainly to the non-use of ancillary techniques.

While we align with the Sydney system’s recommendation to identify the nosologic entity and integrate ancillary tests in the diagnostic process, our approach in the review was broader. In Fig. 10’s diagnostic algorithm, we referenced “sufficient features for malignancy,” intending to encompass all feasible test findings, including morphology, immunocytochemistry, and molecular tests. This was not explicitly stated, but it was implied [2].

Our focus initially was on the morphological features of lymph node fine needle aspiration cytology (FNAC), acknowledging that ancillary tests are not always accessible. In Korea, for instance, excisional biopsy on lymph nodes post-FNAC is common, with most ancillary testing conducted on histologic sections. This preference stems from the rapid and cost-effective nature of excisional biopsies specifically in Korean medical environment. Korean clinicians and patients often opt for confirmatory diagnoses involving more comprehensive sample analyses.

Rapid advancements in molecular pathology have undoubtedly enhanced lymphoma understanding and treatment. However, many countries still struggle to fully incorporate these tests in cytodiagnostics and it sometimes causes subsequent confusion during the diagnostic process in the clinical practice. The study by Gupta et al. [4], also applying the Sydney system, reflects this limitation in India, as ancillary techniques were scarcely employed. Recent detailed molecular research on lymphoid neoplasms has divided the academic community, resulting in the development of two distinct classification systems on histologic diagnosis: the 5th edition of the World Health Organization classification (WHO-HAEM5) and the International Consensus Classification (ICC), which brings many confusions to the practicing pathologists in the daily practice [5]. On the other hand, the Sydney system for cytoplogic diagnosis, in our view, is versatile, accommodating both contexts where molecular pathology is feasible and scenarios reliant solely on morphological evaluation [3,6].

We concur with Caputo et al.’s observations [1] and aimed our review to enrich the diagnostic process, focusing on morphological details. Our comprehensive algorithm, incorporating various morphological features, seeks to streamline and improve diagnostic accuracy. In scenarios where specimen quantity limits further testing, or when additional molecular tests are impractical due to cost, infrastructure, or even sociocultural constraints,
our approach offers direct support to cytopathologists. Hopefully, further validation studies on the Sydney System using real cases of lymph node FNAC in Korea or Asian population should be followed for the reconfirmation of the clinical efficacy as proven in previous studies [4,6]. Looking forward, we anticipate that advancements in digital cytopathology and artificial intelligence image analysis will enhance cancer diagnosis, subtype differentiation, and molecular mutation prediction in near future, as Caputo et al. also suggested [7-9].

**Ethics Statement**

Not applicable.

**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**

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**References**

What’s new in genitourinary pathology 2023: WHO 5th edition updates for urinary tract, prostate, testis, and penis

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Abstract

The 5th edition WHO Classification of Urinary and Male Genital Tumours (2022) introduced many significant changes relevant to urologic daily practice, mainly to renal tumors which was covered in the What’s New newsletter in September 2022. In this newsletter, we summarize the notable changes to bladder, prostate, testis, and penis based on the 5th edition of the WHO.

URINARY TRACT

Inverted urothelial papilloma

• This is reserved for almost exclusively inverted lesions and continues to be in a separate section.
• Use of the descriptor “inverted” in other papillary tumors with inverted histology (non-invasive papillary urothelial carcinoma and papillary urothelial neoplasm of low malignant potential) is discussed within each respective section in the WHO.

Non-invasive papillary urothelial carcinoma, low-grade

• Papillary urothelial hyperplasia and urothelial proliferation with undetermined malignant potential (tented architecture with short, non-branching papillae covered by mildly atypical urothelium) are no longer regarded as distinct entities but are considered early low-grade non-invasive papillary carcinoma.

Invasive urothelial carcinoma

• Presence of TERT promoter mutations can help to: 1) distinguish urothelial carcinoma from a non-neoplastic proliferative process and 2) establish urothelial origin.
• Advanced urothelial carcinoma with FGFR3 alterations may be eligible for treatment with anti-FGFR agents, while mutations in ERCC2 and other DNA damage repair genes may determine those likely to benefit from cisplatin-based chemotherapy.
• Predictors of response to immune checkpoint inhibitors include PDL1 expression in tumor and host immune cells, tumor mutation burden, and microsatellite instability/mismatch repair defect status.
• Terminology is standardized to charac-
terize genetic alterations as “variants,” distinct morphologies as “histologic patterns,” and unique morphologies with prognostic significance as tumor “subtypes.”

- Muscle-invasive bladder carcinoma is divided into 6 molecular subgroups with differing prognosis: luminal-papillary (24%), luminal non-specified (8%), luminal-unstable (15%), stroma-rich (15%), basal-squamous (35%), and neuroendocrine-like (3%).

**PROSTATE**

**Prostatic intraepithelial neoplasia**

- Low-grade prostatic intraepithelial neoplasia has no increased risk of cancer detection and it is not possible to reliably differentiate from benign glandular hyperplasia; thus it was removed as a reportable entity.

**Intraductal carcinoma of the prostate (IDC-P)**

- The cribriform growth pattern of high-grade prostatic intraepithelial neoplasia (HGPIN) is no longer regarded as a distinct entity but rather a spectrum of intraductal proliferation that falls between HGPIN and IDC-P; it is increasingly being referred to as atypical intraductal proliferation (AIP), although there is no consensus on the best terminology.
- IDC-P may represent two distinct entities: 1) the majority are late events associated with invasive high-grade carcinoma spreading into non-neoplastic prostatic ducts and acini and 2) a small subset which arises from a HGPIN precursor and may represent carcinoma in situ.
- The presence of IDC-P should be reported but whether to incorporate areas of IDC-P into Gleason grading remains controversial, with diverging recommendations by leading societies.

**Prostatic acinar adenocarcinoma**

- PIN-like carcinoma is now discussed as a subtype of acinar adenocarcinoma (Fig. 1). It generally has favorable behavior and has the recent distinctive molecular finding of frequent activating mutations in the RAF/RAS pathway.
- Prostate cancers harboring homologous recombination repair defects may respond to poly (ADP-ribose) polymerase (PARP) inhibition, while those with mismatch repair defects may respond to immune checkpoint inhibitors.

**Adenoid cystic (basal cell) carcinoma of the prostate**

- Adenoid cystic carcinoma of the prostate is now the preferred diagnostic term, renamed from basal cell carcinoma, to avoid confusion with the skin tumor.

**TESTIS**

**Noninvasive germ cell neoplasia**

- Gonadoblastoma has been moved from the category called “tumor containing both germ cell and sex cord-stromal elements” into the category “noninvasive germ cell neoplasia”.

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*Fig. 1. PIN-like prostatic adenocarcinoma. A. It is comprised of large discrete glands with flat or tufted pseudostratiﬁed epithelium. B. Immunostaining with HMWCK highlights lack of basal cells in PIN-like carcinoma focus.*
Germ cell tumors derived from germ cell neoplasia in situ

- Teratoma is regarded as containing a somatic-type malignancy if the component that resembles a neoplasm has overgrowth of at least a 5 mm focus (consistent with the previous definition of 1 field at 4x magnification).
- Somatic malignancy composed of immature neuroectoderm was formerly referred to as primitive neuroectodermal tumor (PNET) but has been renamed to teratoma with embryonic-type neuroectodermal tumor (Fig. 2). This helps avoid confusion with the unrelated tumor, Ewing sarcoma.

Germ cell tumors unrelated to germ cell neoplasia in situ

- Well-differentiated neuroendocrine tumor is renamed testicular neuroendocrine tumor, prepubertal-type.

Sex cord stromal tumors

- The recently proposed Leydig cell tumor Scaled Score (LeSS), in which Leydig cell tumor is classified as high or low risk based on size, mitoses, necrosis, infiltrative pattern, and vascular invasion, is mentioned in the WHO but is noted to require further validation (Fig. 3).
- Leydig cell tumor can be FH deficient and this may merit FH testing, particularly in high risk tumors in which mutations may occur more frequently.
- Sertoliform cystadenoma of the rete testis has been moved from being classified as an adenoma within the tumors of the collecting duct and rete testis, to being a part of Sertoli cell tumor.
- The signet ring pattern of Sertoli cell tumor is now listed as a separate tumor called signet ring stromal tumor (Fig. 4). It is composed of signet ring cells which do not contain mucin, is positive for vimentin and β-catenin, and is negative for inhibin. These tumors appear to have indolent behavior.
- Myoid gonadal stromal tumor has been changed from a provisional entity to a confirmed entity. This tumor features densely packed, bland spindle cells positive for SMA and S100. Reported tumors have indolent behavior.

Paratesticular mesothelial tumors

- Well-differentiated papillary mesothelioma is renamed to well-differentiated papillary mesothelial tumor.
The terminology for squamous cell carcinoma groupings has been changed from non-HPV-related to HPV-independent and from HPV-related to HPV-associated.

- Papillary basaloid carcinoma, warty basaloid carcinoma, pseudohyperplastic carcinoma, pseudoglandular carcinoma, carcinoma cuniculatum, and mixed squamous cell carcinoma have been removed as distinct subtypes.
- Pseudohyperplastic and pseudoglandular patterns are now a part of squamous cell carcinoma, usual type.
- Carcinoma cuniculatum pattern is now a part of verrucous carcinoma.

**Meet the Authors**

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